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Characterization of Murine Cytomegalovirus dUTPase homolog, M72, and investigations into novel interacting host factors

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Characterization of Murine Cytomegalovirus dUTPase homolog, M72, and investigations into novel interacting host factors

By

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Dedication

To my father Ramjee Vishwanathan, for your unflagging efforts in filling my life with books and role models. Books became my most cherished friends and you, the best role model I could have ever asked for!

And

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Abstract

Characterization of Murine Cytomegalovirus dUTPase homolog, M72, and investigations into novel interacting host factors

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Abstract: Human cytomegaloviruses (HCMV), a beta herpesvirus, presents a challenge in terms of morbidity and mortality associated with immunocompromised patients and congenital infections. One approach to tackle issues associated with HCMV infection is to understand multiple facets of host-pathogen interactions. However, since herpesviruses are species specific, it becomes imperative to utilize small animal model systems to investigate in the context of natural host. I utilized a genetically and biologically related Murine cytomegalovirus (MCMV) with mice as the small model system. For my dissertation research, I focused on the MCMV dUTPase homolog M72. The dUTPase homologs in the herpesvirus family are classified as core genes and significant roles are ascribed to them. However, little was known specifically about the role of dUTPase homologs among beta herpesviruses. Human cytomegalovirus (HCMV) UL72 and murine cytomegalovirus (MCMV) M72 were designated as dUTPases based on limited sequence and positional homology. I found that M72 is not enzymatically active as a dUTPase and is expressed as a leaky-late gene product with multiple protein isoforms. Additionally, M72

augments virus replication *in vitro* and in the acute phase *in vivo*. To begin to understand M72 function, I took a proteomics approach and identified interacting host protein partners. I identified and confirmed interaction of M72 with the eukaryotic chaperonin tailless complex protein-1 (TCP-1) ring complex (TRiC) or chaperonin containing tailless complex polypeptide 1 (CCT). Accumulating biochemical evidence indicates M72 forms homo-oligomers and is a substrate of TRiC/CCT. To explore the role of M72 beyond protein folding, I also identified components of Carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) or CCR4-NOT complex, including the 182-kDa Tankyrase 1 binding protein (TAB182) as M72 candidate interacting proteins. My current work suggests that CCR4-NOT complex subunit 1 (CNOT1) is necessary for MCMV replication. Additionally, M72 mediates its own function at least partially via CNOT1 during virus replication.

Taken together, this research provides the first evidence of a beta herpesvirus dUTPase homolog's contribution to viral replication. My dissertation research has helped uncover host proteins novel for herpesviruses as interacting partners. In addition, one of these host factors, CNOT1, contributes to M72 mediated function.

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Chapter 1: Introduction[1]

1.1 Overview

In this chapter, I will review the following topics relevant to my dissertation – a general overview of the family of Herpesviruses, the virus of my particular interest, Human Cytomegalovirus (HCMV) and small animal model systems to study HCMV. I will then discuss the model organism of my interest Murine Cytomegalovirus (MCMV), focus on the gene family of relevance to my dissertation, herpes viral dUTPase homologs, and specifically focus on the MCMV designated dUTPase homolog M72. I will subsequently review some novel host factors that I identified as M72 interacting proteins – 1) host chaperonin tailless complex protein -1 (TCP-1) ring complex (TRiC)/chaperonin containing tailless complex polypeptide 1 (CCT) and 2) Carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) or CCR4-NOT transcriptional complex.

1.2 Herpesviruses

1.2.1 Introduction to Herpesviruses

Herpesviruses are large enveloped viruses. The name is derived from the Greek word *herpein*, meaning, "to creep", referring to the recurring infections due to herpetic sores in the mouth and groin. Herpesviruses are ubiquitous in nature and infect both vertebrate and non-vertebrate species. To date more than 130 herpesviruses are characterized, and eight have been routinely isolated from humans [2]. In general, viruses in this family are highly species specific with the natural host range of individual viruses

[1] Portions of section 1.7.2 reviewing 'Herpesviral dUTPase homologs' are from my paper [1]. Gopal, S., et al., *Murine cytomegalovirus M72 promotes acute virus replication in vivo and is a substrate of the TRiC/CCT complex.* Virology, 2018. **522**: p. 92-105. I wrote the initial manuscript and primarily Jason W. Upton (JWU) edited it.

restricted to a single species. Herpesviruses display both lytic and latent stages in their life cycles. Disease manifestations in humans range from mild fever and rash to cancer across this family.

1.2.2 Classification of Herpesviruses

The order *Herpesvirales* is classified into three families of which the family *Herpesviridae* includes viruses infecting mammals, birds and reptiles. *Herpesviridae* are studied most commonly and divided into three subfamilies: *Alphaherpesvirnae*, *Betaherpesvirinae* and *Gammaherpesvirinae* [3, 4]. This classification is based on genomic sequences and shared biological properties. It also serves the purpose of explaining evolutionary relatedness and distinguishing unique properties (Table 1.1) [2, 4].

Subfamily	**Properties**
Alphaherpesvirinae	1. Very short (hours) infection cycle 2. Establishment of latency in sensory neuron
Betaherpesvirinae	1. Very long (days) reproductive cycle 2. Establishment of latency in secretory glands, reticuloendothelial cells and kidneys
Gammaherpesvirinae	1. Primary infection is following by periodic reactivation 2. Latency in B cells 3. Unique due to its association with human malignant tumors

Table 1.1: General properties of subfamilies of *Herpesviridae*.

Table showing properties of subfamilies of herpesviruses [2, 4].

1.2.3 Structure of Herpesviruses

Membership in the *Herpesviridae* family is dependent on virus morphology. Virions are spherical and consists of four components – core, capsid, tegument, and envelope. The core consists of a single copy of double stranded linear DNA molecule packed tightly. Cores are surrounded by an icosahedron capsid with 162 capsomeres, consisting of 12 pentons and 150 hexons [5]. The tegument is an amorphous layer of proteins surrounding the capsid. The outermost layer consists of the viral envelope, studded with viral membrane glycoproteins and some cellular proteins. The composition of the tegument and envelope varies widely across the family.

1.2.4 Infection cycle in Herpesviruses

After initial infection of host cells, viral transcription, genome replication and capsid assembly occurs in the nucleus. Gene expression is temporally regulated with a sequential cascade of: (1) immediate-early genes, which make regulatory proteins; (2) early genes, which make enzymes for DNA replication; (3) late genes, which generate structural proteins. Virions bud out from the nucleus or the endoplasmic reticulum, and the tegument and envelope are acquired during this process. The virions are transported to the cell membrane, and the host cell dies as the virions are released [2].

Alternatively, latent virus can be maintained during which no replication occurs, and only few viral proteins are expressed. Latency is broadly characterized by – (1) viral genome maintenance in infected cells and (2) avoidance of the immune system and cell death. In the event of cellular or environmental stress, latently infected cells can reactivate from this state and express gene products sequentially [6].

1.3 HUMAN HERPESVIRUSES

Nine herpesviruses infect humans and are spread across all three subfamilies. Prevalence of herpesviruses is determined by serology [7] and it varies depending on the type of virus. These viruses are believed to have co-evolved over a long period of evolutionary history with their hosts. Hence, their interactions with the host are typically benign compared to their more zoonotic counterparts like Severe Acute Respiratory Syndrome (SARS) coronavirus, Ebola and Avian influenza. Thus, a healthy individual will have minor symptoms due to infection with herpesviruses. Chickenpox blisters and herpes cold sores are exceptions to this. Disease manifestations include mild fever, rash, and infrequently cancer (Table 1.2). Human herpesviruses are typically restrained by an immunocompetent host after which they will become latent. Alpha herpesviruses become latent in neurons, whereas cells of monocyte lineage are believed to be involved in the latency of beta herpesviruses while gamma herpesviruses become latent in lymphocytes [6]. Transmission of human herpesviruses is usually by close contact with bodily secretions and typically happens early in life [8].

Currently there are vaccines for prevention of Varicella-zoster virus (VZV) – a live attenuated vaccine for prevention of varicella (or chicken pox) and a therapeutic vaccine for herpes zoster (or shingles) [9, 10]. Other promising vaccine candidates for Herpesvirus Simplex Virus (HSV)-1, 2 and HCMV are in several stages of clinical trials. Of these, progress towards the HCMV vaccine is a very active area of research with focus on a major HCMV glycoprotein gB as a candidate immunogen [11]. Development of Epstein-Barr virus (EBV) vaccines has been slower, yet EBV glycoprotein gp350 recombinant protein based candidate vaccines are in different phases of trials [12].

Some of the human herpesviruses are amenable to antiviral treatment. Acyclovir is preferred for HSV and VZV infections in immunocompromised hosts [13]. Valaciclovir and Famciclovir are licensed for shingles in the elderly [14], whereas Ganciclovir and Foscarnet are the treatment of choice for HCMV retinitis [15].

Name	Subfamily	Common Name	Disease	Transmission route
HHV 1	alpha	Herpesvirus Simplex Virus 1 (HSV-1)	Genital/oral sores, encephalitis,	Intimate contact (Oral or sexual route)
HHV 2	alpha	Herpesvirus Simplex Virus 2 (HSV-2)	Genital/oral sores, encephalitis,	Intimate contact (Oral or sexual contact), nosocomial for infants
HHV 3	alpha	Varicella-zoster virus (VZV)	Chickenpox, shingles	Respiratory, intimate contact (including sexual)
HHV 4	gamma	Epstein-Barr virus (EBV)	Infectious mononucleosis, Hodgkin's lymphoma, Burkitt's lymphoma	Intimate contact, transfusions, tissue transplant, congenital
HHV 5	beta	Cytomegalovirus (CMV)	CMV mononucleosis, CMV retinitis, congenital cytomegalic inclusion disease	Saliva, blood, urine, breast milk, solid-organ transplants, congenital
HHV 6A and 6B	beta	Roseola virus	Roseola infantum (sixth disease or exanthem subitum)	Respiratory and intimate contact ?
HHV 7	beta	Roseola virus	Roseola in infants, encephalopathy	Unknown
HHV 8	gamma	Kaposi sarcoma-associated Herpesvirus (KSHV)	Kaposi's sarcoma, Multicentric Castleman's disease, Primary effusion lymphoma	Intimate contact

Table 1.2: Human Herpesviruses and their disease manifestations.

Table summarizing the family of human herpesviruses [3, 16].

1.4 HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV) or *Human Herpesvirus 5* (HHV-5) and other animal cytomegaloviruses belong to the sub-family *Betaherpesvirinae* and genus *Cytomegalovirus*. HCMV is a significant human pathogen, especially in developed countries. The name cytomegalovirus is derived from the characteristic enlargement of infected cells (cytomegaly) and presence of nuclear inclusion bodies (typically aggregates of stable proteins).

1.4.1 History of Cytomegalovirus

The German scientist Hugo Ribbert first observed HCMV in 1881 in tissue sections of kidney and parotid gland but was not able to identify the etiological agent. Subsequent German scientists in 1904 described, 'eccentrically-placed nucleus surrounded by a halo' in sections of kidneys, liver and lungs of fetuses, which was interpreted as a protozoan infection. These are probably the first descriptions of cytomegalic inclusions in published literature, but the viral etiology of these observations was not yet known (summarized by Ho Monto, 2008 [17]). Subsequent findings of similar inclusion bodies from herpes zoster lesions [18, 19] led to suspicions of a viral etiology from a group of related viruses (now known as Herpesviruses). At this point in scientific history, though the general concept of virus was accepted, the very nature of virus itself was not clearly understood. Later, observations of inclusion bodies from 25 rare cases of congenital infection characterized by petechiae, hepatosplenomegaly and intracerebral calcification led to the introduction of the term - generalized cytomegalic inclusion disease (CID). With the advent of mammalian cell culture technique [20], the challenge of culturing HCMV was tackled. Dr. Margaret G. Smith made pioneering efforts in isolating cytomegaloviruses. Eventually, three scientists – Margaret G. Smith, Wallace P. Rowe and Thomas H. Weller independently isolated

cytomegaloviruses from 1956–57. They exchanged the isolated agents with each other and collaborated to confirm the similarity of agents [21-23]. Thomas H. Weller suggested the name cytomegalovirus [24, 25].

1.4.3 Characteristics of HCMV

HCMV structure includes features typical of the herpesvirus family as illustrated in Figure 1.1. The virion is about 230 nm in diameter and consists of an icosahedral capsid, a tegument and a trilaminate membrane envelope. The capsid encloses a linear, double-stranded DNA genome approximately 236 kb in size (in wild type virus) [26], which is the largest among human herpesviruses. The capsid is approximately 110 nm in diameter and made up of four integral protein species - pUL46, pUL80.5, pUL85 and pUL104 – in the framework of 162 capsomeres. The tegument is an amorphous layer, approximately 50 nm thick; however, some structuring of tegument proteins close to the capsid has been observed [27]. This layer includes about 79 viral proteins, although only 35 are found abundantly [28, 29]. The outermost envelope is about 10 nm thick, and contains about ten abundant viral proteins. Less-abundant viral and host proteins, phospholipids, polyamines and small RNAs are also included in the composition of the tegument and envelope [30].

Particle composition varies depending on source, method of preparation and nature (sensitivity) of analysis. Apart from virions, five other types of viral particles have been isolated from CMV infected cells as summarized in Table 1.3 [30, 31]. Briefly A-, B- and C- type capsids can be obtained by lysing CMV infected cells with NP-40, whereas non-infectious enveloped particles (NIEP) and Dense Bodies (DB) can be recovered from culture supernatants of CMV infected cells. In general, HCMV is sensitive to lipid-

dissolving agents, low pH conditions and heat. It is not stable at -20ºC but must be stored at -80ºC to maintain infectivity [26].

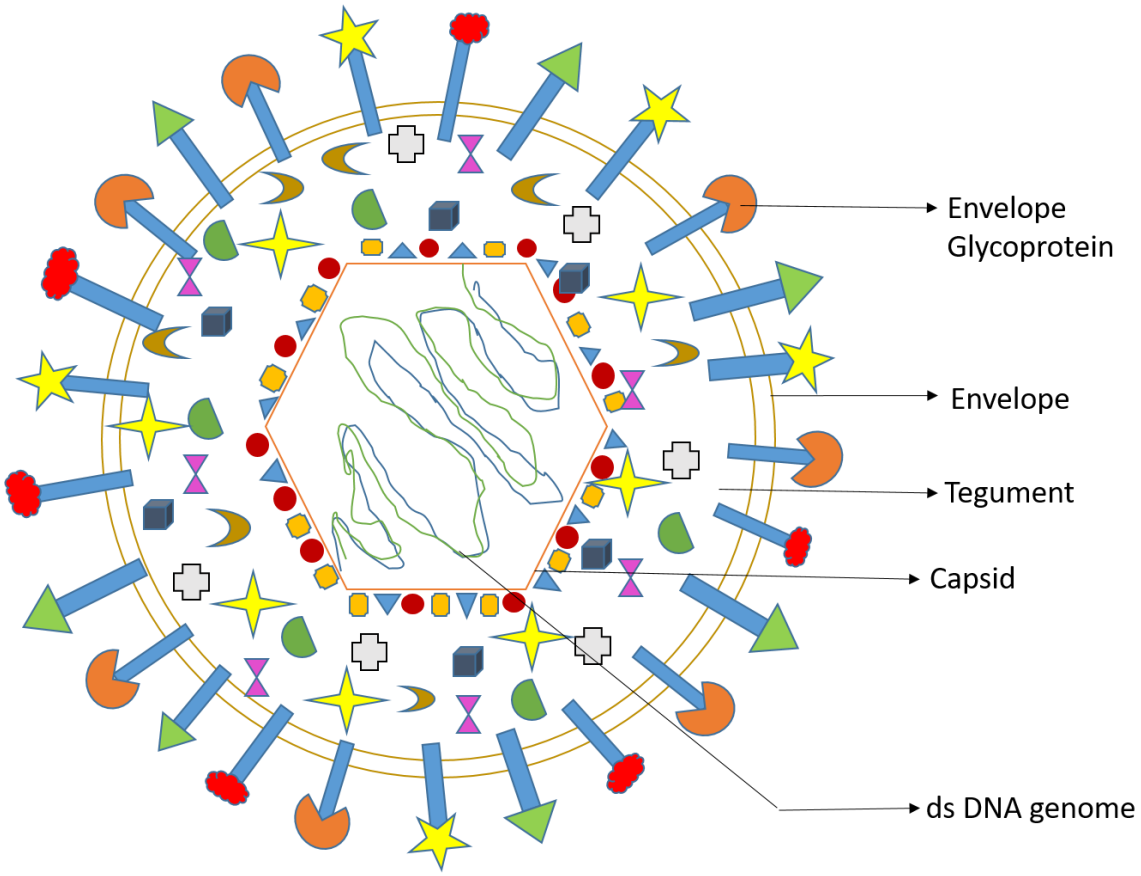


Figure 1.1: Schematic of HCMV structure

This figure illustrates the general structure of Cytomegaloviruses (HCMV and other animal CMV). The double-stranded linear DNA genome is tightly packed inside an icosahedral capsid surrounded by a tegument layer with several proteins. The tegument is bound by the envelope decorated with glycoproteins, which mediate viral entry into host cells.

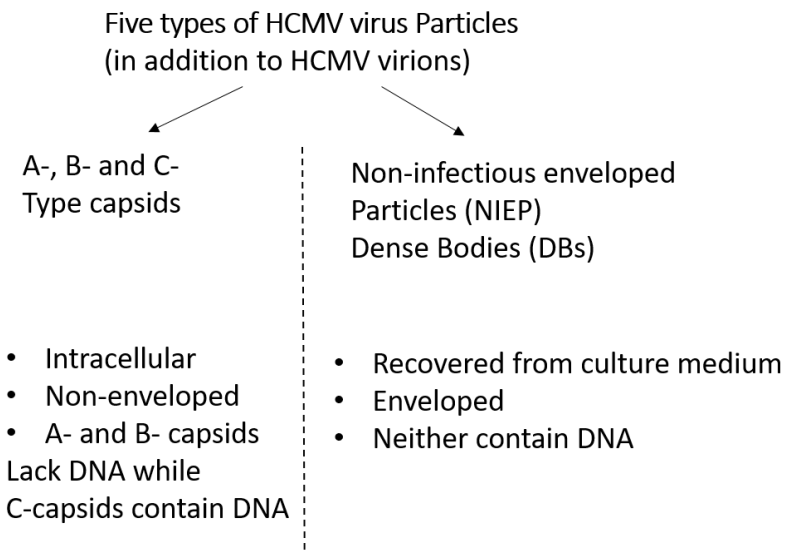


Figure 1.2: Five types of HCMV particles.

Different types of HCMV particles [30, 31].

1.4.4 HCMV Replication

HCMV infects a wide variety of cells including fibroblasts, endothelial cells, smooth muscle cells, epithelial cells, monocytes/macrophages, although human fibroblasts constitute the major cell target that aids replication of virus [32]. The steps involved during virus multiplication are illustrated in Figure 1.2. Multiple cellular receptors like epidermal growth factor receptor (EGFR) [33], platelet derived growth factor receptor alpha (PDGFRα) [34] and bone marrow stromal cell antigen 2 (BST2)/Tetherin [35] have been described in literature. Depending on cell type and strain of virus (laboratory strain AD169, Towne or clinical strain TR) both fusion at the plasma membrane and endosomal acidification can facilitate entry [36-38]. Viral glycoproteins gB [39], gM/gN [40], gH/gL [41] and gO [42] or UL128-UL131 [43-45] are some important examples that mediate entry depending on cell type. After interaction of viral glycoproteins with the host cell, and initial fusion events, the capsid is deposited in the cytoplasm. Tegument proteins are also released in this step into the cytoplasm and mediate roles during early stages of infection [46]. Within a short time, the capsid is transported to the nucleus primarily via the microtubule network [47]. The capsid docks at the nuclear pore complex and the genome is released into the nucleus where viral transcription, genome replication and encapsidation occur. This cascade of events occurs in a sequentially and temporally regulated manner, similar to other herpesviruses. Immediate-early genes are transcribed first, and they do not require *de novo* protein synthesis. These gene products facilitate/regulate the early genes, leading to DNA replication. Subsequently, late genes are made [48, 49]. The viral capsid is assembled inside the nucleus and transported to the cytoplasm where virion assembly and maturation is orchestrated by the cytoplasmic viral assembly complex (AC). The AC manipulates components of the cellular secretory process including the endoplasmic

reticulum, Golgi complex and the endosomal machinery. The viral envelope and tegument is acquired in the AC, and mature virions or dense bodies are released in the extracellular space [50-53].

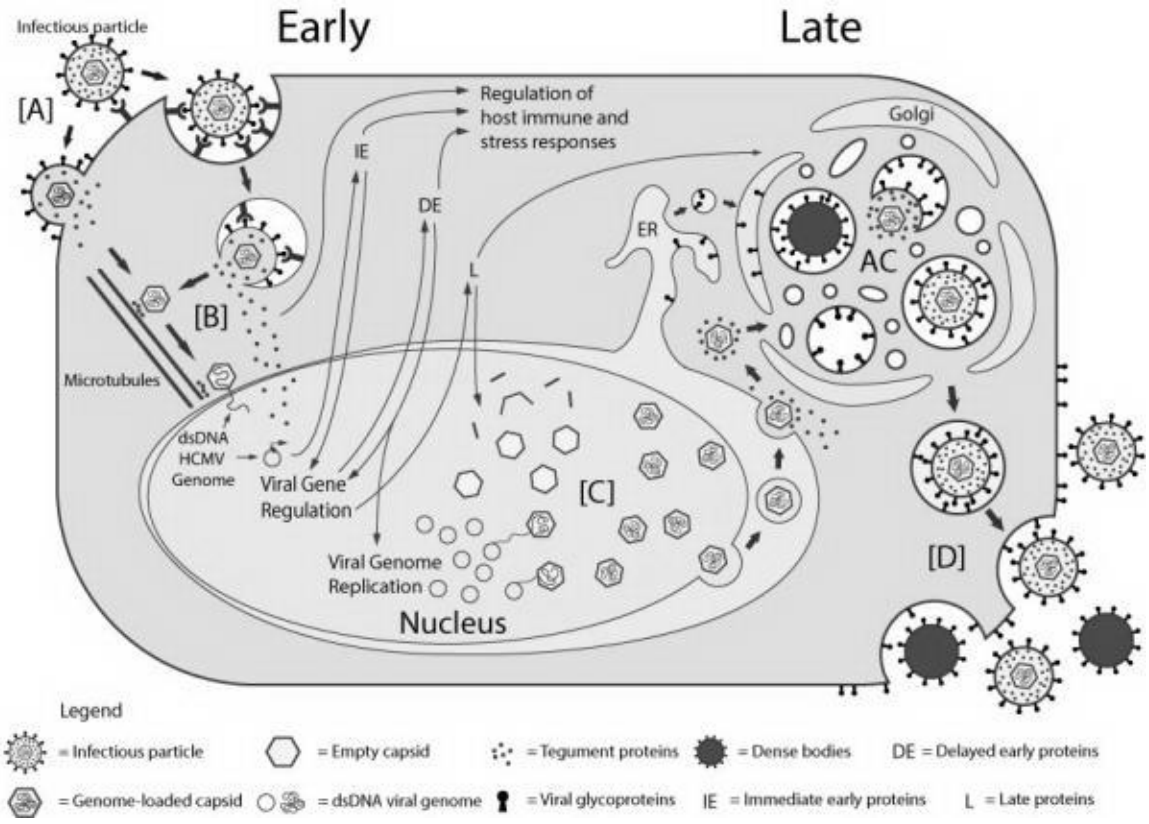


Figure 1.3: HCMV Replication

A) Interaction of infectious viral particles with host receptors facilitates entry into the cell, delivering capsid and tegument proteins into the cytosol. B) Microtubules mediate transport of capsid to the nucleus, where genome is delivered and circularized. Tegument proteins regulate host responses and initiate the sequential cascade of Immediate Early (IE), Delayed Early (DE) and Late (L) genes. DE genes initiate viral DNA replication. C) Late gene expression initiates capsid assembly in the nucleus, which egress to the cytosol. Tegument proteins associate with capsids in the cytosol and are trafficked to viral assembly complex (AC), which contains components of endoplasmic reticulum (ER), Golgi apparatus and endosomal machinery. The capsids acquire tegument and viral envelope and bud into intracellular vesicles in the AC. D) Both enveloped infectious particles and non-infectious dense bodies are released. (Modified from Beltran and Cristea, 2014 [54])

1.4.5 HCMV Infection and Infectious Disease

Primary HCMV infection (first infection in life) in immunocompetent individuals is mostly unnoticed or characterized by low-grade fever, mononucleosis-like syndrome, fatigue and sore throat. Very rare instances of severe clinical symptoms occur due to primary HCMV infection in immunocompetent individuals [55-57]. Primary HCMV infection is typically, acquired through direct contact with the genitourinary, respiratory and upper alimentary tract [58, 59]. Upon infection, the virus is tackled by a robust host immune system. However, HCMV is not eliminated, but rather becomes latent for the lifetime of the individual. Latency is also asymptomatic with intermittent reactivation [49].

The challenges with HCMV arise in immunocompromised individuals, *e.g.*, transplant recipients, HIV/AIDS patients or if the immune system is immature resulting in cases of congenital HCMV infections. These challenges could be due to primary HCMV infection in these individuals or reactivation of HCMV from latency or re-infection by another strain of HCMV [60]. In this sense, HCMV is an opportunistic pathogen.

Transplant recipients undergoing hematopoietic stem cell transplant (HSCT) or solid-organ transplant (SCT) are under immunosuppressive drug(s) regimen, which makes them prone to HCMV complications. HCMV-seropositive (have IgG antibodies against CMV specific antigens) donors can transmit virus to recipients, independent of serological status of recipient [61]. Additionally, re-activation and re-infection can contribute to morbidity and mortality post-transplantation [62]. Pneumonitis, gastrointestinal ulceration, hepatitis, retinitis and death are common manifestations of clinical disease due to HCMV infection post-transplantation. Moreover, HCMV promotes graft rejection and accelerates atherosclerosis in patients who underwent heart or lung transplant [63]. Currently, these complications can be controlled via treatment with Ganciclovir [64].

AIDS patients are another subset of the population facing HCMV complications due to immunodeficiency. HCMV retinitis and other end-organ disease are common challenges in these patients [65]. In certain cases, even after controlling HIV viral load and CD4 counts, HCMV results in increased mortality in these patients [66]. Combined administration of intraocular and systemic Ganciclovir is a therapeutic treatment of choice [67].

Congenital HCMV infection is defined as infection of the immature fetus due to maternal infection via the placenta within two weeks of birth [68]. In developing countries, virtually 100% of women entering childbearing age are HCMV seropositive, and these women are susceptible to reactivation of latent virus or reinfection with new strain, both of which can be transmitted via placenta to the fetus. In developed countries, many antenatal women are seronegative for HCMV making them susceptible to HCMV primary infection [60, 69, 70]. Symptomatic children have classic features at birth whereas others are asymptomatic at birth but develop symptoms upon follow up. Hepatosplenomegaly, microcephaly, thrombocytopenic purpura, and sensorineural hearing loss (SNHL) are typical features of HCMV infection at birth. Upon follow-up, SNHL and/or neurodevelopmental delay appear as symptoms, which continue progressively [71].

1.4.6 HCMV Prevalence

Globally, HCMV prevalence is assessed based on serological status and is estimated to be 30–100%. In developed countries, *e.g.*, USA, Europe and Australia, HCMV occurrence is between 36–76%. In developing countries such as Brazil, sub-Saharan Africa and India, seroprevalence reaches almost 100% [72]. The occurrence of HCMV infection

and development of related complications (HCMV disease) varies depending on the broad groups (discussed earlier in Section 1.4.5) under consideration.

Transplant recipients can be broadly categorized as – hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) patients. Among HSCT cases, both groups – allogeneic (stem cells derived from human leukocyte antigen (HLA) matched donor) and autologous (stem cells from the same individual) recipients develop HCMV disease. In the absence of any prophylactic treatment, HCMV incidence varies based on factors like type of immunosuppressive regimen, type of transplantation, HCMV serological status match between donor and recipient, and any other illnesses. About 30% of allogenic HSCT recipients and 5% of autologous HSCT recipients develop HCMV disease [62]. Among SOT recipients, the occurrence of HCMV disease is about 18–29% in post liver transplants [73], and as high as 70% after kidney transplants [74]. A large multicenter study following heart transplant patients found HCMV is the most common infecting organism, accounting for 26% of all infections [75].

Congenital HCMV infection results in a substantial burden of disease globally but the most reliable population-based estimates are from the USA. About 28,000 (0.4% - 0.7%) babies are born each year with congenital HCMV infection; 12.7% are symptomatic at birth whereas 13.5% of asymptomatic babies develop symptoms upon follow-up [71, 76, 77]. A systematic literature review of studies in developing countries (defined by the International Monetary Fund) prior to 2013 estimated a HCMV birth prevalence of 0.6–6%. Maternal seroprevalence was between 84-100%. HCMV-associated sequelae were not documented in most cases [78]. Other reviews have estimated similar HCMV birth prevalence in developing countries [70, 77, 79] suggesting that the burden of congenital HCMV in developing countries is underappreciated and, in fact, highly neglected. These

data also question the dogma in the field that children born with congenital HCMV to seropositive mothers have normal clinical outcomes.

In summary, understanding HCMV prevalence highlights the global burden - apparent or silent.

1.5 MODEL SYSTEMS TO STUDY CMV

CMVs are associated with many animal species and utilized to study natural infections. Rodents (mouse, rat, hamster and guinea pig), rabbits, pigs, horses, bovid animals and primates (*Rhesus macaque* and chimpanzees) are examples of animal species infected by CMV. Murine CMV (MCMV) has been used to study the susceptibility of mice to infection, effect of CMV on embryonic development, infection of the inner ear, ocular infections, and cardiac infection. MCMV has also been utilized to assess latency and reactivation [80].

Cavid herpesvirus type 1 or Guinea Pig CMV (GPCMV) is most commonly utilized to understand issues related to *in utero* CMV infections. Guinea pig placental histology is much closer to humans than that of mice and GPCMV can cross the placenta (unlike MCMV) and lead to fetal infection and disease. This facilitates evaluation of issues such as timing of transplacental transmission, contribution of placenta in assisting or averting CMV, how maternal immunity affects the outcome of fetal infection and disease pathogenesis in newborns [81].

The Rat CMV (RCMV) genome has been sequenced [82] and shown to be amenable to mutagenesis [83]. RCMV is now becoming a relevant model to study CMV associated transplant issues, e.g., accelerated allograft rejection and development of vascular diseases [84].

Porcine CMV (PCMV) has recently garnered attention due to the use of pig organ grafts in humans (xenotransplantation). PCMV may be reactivated in grafts, leading to development of invasive disease. Hence, studies of PCMV susceptibility to antiviral drugs to prevent transmission in pig xenograft organs are valuable and need to be explored in this model system [85].

Chimpanzee CMV (CCMV) has the most homology to HCMV [86]. However, chimpanzees are a protected species and not readily available, making their use limited as an animal model system. The other non-human primate model is *Rhesus macaque* (RM), which harbors Rhesus CMV (RhCMV) and is more readily available as a significant model system. *Rhesus macaques* are typically used to evaluate the effect of antivirals in the context of bioavailability and toxicity because they are much closer to humans than small animal models. RhCMV is also utilized to characterize immune responses in RM. Since HCMV is an opportunistic disease in AIDS patients, RMs are also useful to evaluate RhCMV disease progression in Simian immunodeficiency virus (SIV)-infected monkeys [87].

Rodents are easier to maintain than larger animals. In addition, inbred mouse strains allow minimal individual variation. Strain specific differences in susceptibility of mice to MCMV infection [88] have helped dissect out intricate immune mechanisms. Therefore, MCMV has been used extensively to understand the biology of CMV and evaluate host factors involved in virus replication. Over past decades, significant characterization of viral genes involved in MCMV replication and immune responses by the host has been achieved. In comparison, there is relatively less understanding of RCMV or GPCMV. Additionally, the advent of genetic manipulation tools like CRISPR/Cas9 has improved evaluation of host factors in mice [89]. HCMV and MCMV share the type of pathology seen as well as

the cells and organs that are infected [88]. However, having described all the advantages of the mouse model, it is important to mention that MCMV does not cross the placental barrier, limiting [90] its use for studies relating to transplacental transmission of CMV.

In recent years, advancements in development of humanized mouse models by transfer of xenografts have been used to evaluate HCMV [91]. Yet, there are limitations to this system and so MCMV continues to be an important small animal model system to study CMV biology.

1.6 MURINE CYTOMEGALOVIRUS (MCMV)

MCMV belongs to the family of beta herpesviruses and is a large double-stranded DNA virus with host specificity for mice. Most of the HCMV structural (described in Section 1.4.3 earlier) features are shared with MCMV, including an outer lipid envelope, amorphous tegument and icosahedral nucleocapisd. Similar general mechanisms of the HCMV and MCMV replication cycles involving a temporally regulated cascade of viral genes are also common (described earlier in Section 1.4.4). The genome of HCMV and MCMV are both colinear, and many of MCMV proteins share sequence and functional similarity with HCMV. This offers advantages for investigation of the contribution of conserved genes in the context of the natural host. There are 43 core genes evolutionarily conserved among the subfamilies of herpesviruses. Many of these core genes are located in the central region of the genome and play a role in basic virus replication [3]. However, some core genes are relatively uncharacterized. Our focus is on one such MCMV core gene, M72, designated as a 2'deoxyuridine 5'triphosphate pyrophosphatase (dUTPase) homolog.

1.7 HERPESVIRAL dUTPASE HOMOLOGS

1.7.1 Cellular dUTPase

The 2'deoxyuridine 5'triphosphate pyrophosphatase (dUTPase) cellular enzyme is involved in conversion of dUTP to dUMP and pyrophosphatase (Fig. 1.3) thus decreasing dUTP levels in the host cell. This is important for reducing misincorporation of uracil into DNA because most DNA polymerases cannot distinguish between thymine and uracil. In addition, the product dUMP is a substrate for thymidylate synthase enzyme and provides dTTP precursor for the host cell [92, 93]. Given its significant role in cellular nucleotide metabolism, dUTPases are ubiquitous enzymes present in prokaryotes, eukaryotes and viruses [94]. Such enzymes are developmentally regulated with high activity in undifferentiated cells and low level of activity in terminally differentiated cells. The dUTPase expression also varies according to the cell cycle and its levels are elevated in dividing cells and reduced in non-dividing cells [95, 96].

The dUTPase protein consists of five domains designated as Motifs 1–5. The dUTPase fold is a classic jellyroll β-sheet, which forms a homotrimer. The enzyme in most organisms is specific for dUTP as a substrate and not dUDP [94]. Human cells have two isoforms – nuclear and mitochondrial dUTPases. The nuclear isoform is under cell cycle control, whereas the mitochondrial isoform is constitutively active. These isoforms are made utilizing alternative 5'exons [97].

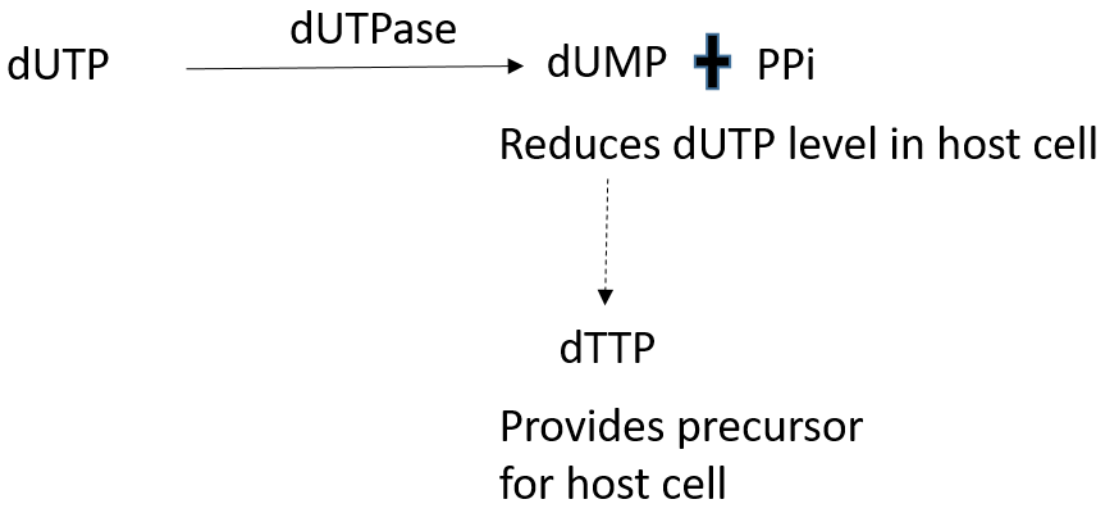


Figure 1.4: Role of Cellular dUTPase enzyme.

Catalytic activity of dUTPase enzyme and its contribution to cellular nucleotide pool [93].

1.7.2 Viral dUTPase homologs

Multiple examples of viral dUTPases exist among retroviruses and DNA viruses [94]. The dUTPase-encoding gene in non-primate lentiviruses is essential for replication in non-dividing cells [98]. Similarly, a feline immunodeficiency virus (FIV) dUTPase mutant displays a reduced viral burden *in vivo*, suggesting a contribution to infection in the natural host [99]. A dUTPase-homolog is present among all three herpesvirus subfamilies - alpha, beta and gamma (summarized in Table 1.4). Among these, alpha and gamma herpesvirus dUTPases are enzymatically active [100].

The Alpha herpesvirus dUTPase is catalytically active but multiple roles independent of its enzyme activity have been described. An initial study showed that an

HSV 1 dUTPase, UL50, null mutant grows to comparable levels as wild type virus [101]. A subsequent study found that an HSV 1 dUTPase mutant is less neurovirulent and exhibits diminished capacity to reactivate from latency [102]. Pseudorabies virus (PRV) dUTPase UL50 was shown to degrade type I interferon receptor by the lysosomal turnover pathway. This activity was independent of dUTPase activity of UL50 [103]. The VZV dUTPase, encoded by ORF8, showed a modestly diminished yield in cell culture [104]. The Simian varicella virus (SVV) dUTPase encoded also by ORF8, grows to almost comparable levels as wild type virus in cell culture and is expressed in the natural host [105]. Among beta herpesviruses, examination of the HCMV designated dUTPase homolog, UL72, revealed that the protein is catalytically inactive and dispensable for replication in cell culture [106].

Among gamma herpesviruses, where the designated dUTPase gene product is enzymatically active, functions independent of their dUTPase activity are documented. EBV encoded dUTPase, BLLF3, contributes to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [107] and promotes the release of pro-inflammatory cytokines in a Toll-like Receptor (TLR) 2-dependent fashion [108]. KSHV ORF54 reduces the expression of a cell-surface ligand, NKp44L [109]. The Murid herpesvirus-68 (MHV-68) dUTPase ORF54 degrades Interferon receptor 1 protein [110]. It was established that HSV 2, HHV 6A, HHV 8 and VZV encoded dUTPases contribute to cytokine activation and modulate dendritic cell activity utilizing *in vitro* assays [111]. Therefore herpesvirus dUTPase homologs have diverse and significant roles in virus biology (Table 1.4). A lack of understanding about the contribution of beta herpesvirus

dUTPase homologs indicates that their study will help uncover novel roles in virus replication.

Virus Subfamily	Virus	dUTPase gene	Catalytic activity	Contributes to *In vitro* replication	Function
alpha	HHV 1/2 (HSV 1/2)	UL50	yes	no	Induces cytokines and modulates function of dendritic cell (DC)
alpha	HHV 3 (VZV)	ORF8	yes	no	Induces cytokines and modulates function of DC
beta	HHV 5 (HCMV)	UL72	no	no	ND
beta	HHV 6A	U45	Not determined (ND)	ND	Induces cytokines and modulates function of DC
Beta	HHV 6B	U45	ND	ND	ND
beta	HHV 7	U45	ND	ND	ND
gamma	HHV 4(EBV)	BLLF3	yes	ND	Activates NFκB and induces cytokines
gamma	HHV 8(KSHV)	ORF54	yes	ND	NKp44L suppression

Table 1.3: Human Herpesvirus dUTPase homologs.

Summary of dUTPase homologs in Human Herpesviruses (Adapted from M. Williams *et al.*, 2017 [112]).

1.8 HOST FACTORS NOVEL FOR CMV BIOLOGY

1.8.1 TRiC/CCT Chaperonin complex

The eukaryotic chaperonin tailless complex protein -1 (TCP-1) ring complex (TRiC)/chaperonin containing tailless complex polypeptide 1 (CCT) referred here as TRiC/CCT is a hetero-oligomeric complex implicated in protein folding in the cell.

1.8.1.1 The Molecular Chaperone System

Chaperones and chaperonins are sub-classes of a distinct family of proteins commonly termed molecular chaperones that are involved in folding polypeptides in the cell but are not components of the structures formed [113]. Chaperones are monomeric, 70–90 kilo dalton (kDa) proteins and strikingly stress-inducible. Some commonly studied examples are the Hsp70 and Hsp90 family of proteins. They include the bacterial proteins DnaK, DnaJ and GrpE; eukaryotic cytosolic protein Hsp72, mHsp70 from mitochondria and BiP in the endoplasmic reticulum as representative examples [114]. In contrast, chaperonins are oligomers of approximately 800 kDa molecular weight that have multiple subunits and are stress-independent. The bacterial GroEL/GroES system and the eukaryotic TRiC/CCT family belong to this sub-class [115].

1.8.1.2 Features of the TRiC/CCT Chaperonin complex

The TRiC/CCT chaperonin is composed of eight paralogous subunits (CCT 1-8) arranged as back-to-back double-rings surrounding a central cavity. The yeast subunits are called CCT (1-8) p whereas, in mammalian cells they are referred to as CCT α, β, γ, δ, ε, ζ, η and θ [116]. A ninth testis specific subunit has been described with homology to CCT ζ and referred as CCT ζ-2 [117]. The eight different subunits are made from eight homologous genes [116]. This complex operates in an ATP-dependent manner with cycles

of conformational changes upon nucleotide binding and hydrolysis that aid opening of the chamber and encapsulation of the substrate [118, 119]. A specific arrangement of the eight subunits surround the ring [120, 121]. Each subunit has an apical, intermediate and equatorial domain. The equatorial domain has nucleotide-binding capacity whereas the apical domain is involved in substrate specificity. The flexible intermediate domain connects the two. The apical domain is diverse in terms of its sequence compared to the equatorial and intermediate domains. This suggests that the eight subunits capture a wide variety of client proteins within the ring [122-124]. CCT is not upregulated during heat shock, indicating a contribution to protein folding in a normal cellular environment [125].

1.8.1.3 Principles of TRiC/CCT Chaperonin complex substrate recognition

The substrate specificity of TRiC/CCT is complex and not well understood. Simpler rules regarding hydrophobicity and sequence features of proteins are not sufficient to explain substrate recognition mechanisms. A combination of in vivo screening and bioinformatics approaches estimated that approximately 6-7% of the eukaryotic cellular proteome is a substrate of the TRiC/CCT complex [126]. Although previous reports had suggested a TRiC/CCT binding sequence motif [127, 128], later analysis failed to identify any consensus sequence common between the identified substrate proteins. Instead, structural features, such as proteins prone to be part of oligomer assembly or proteins with a propensity for β-sheets (and low α helical content) for 35-45 amino acid stretches, were common in substrate proteins. These features along with co-translational association and association with co-chaperone proteins contribute to substrate binding[126]. Additionally, in lieu of single substrate-apical domain interaction, binding with multiple subunits at

discrete sites offers a more stable interaction with substrate. Thus, TRiC/CCT interacts with more diverse sets of proteins compared to GroEL chaperonin in bacteria [129].

1.8.1.4 Host proteins as substrates of the TRiC/CCT Chaperonin complex

Actin and tubulin are two well-characterized substrates of the TRiC/CCT complex [130, 131]. Other examples include the heavy meromyosin (HMM) fragment of skeletal muscle myosin, which complexes with TRiC/CCT in a rabbit reticulocyte lysate (RRL) system [132]. Additionally, some cell-cycle related proteins have been identified as TRiC/CCT substrates. Cyclin E, involved in G1/S phase transition utilizes TRiC/CCT complex to achieve its native structure [133]. Polo-like kinase 1 is a protein, which participates in G_2/M cell cycle progression and associates with TRiC/CCT immediately after translation. Transient knockdown of TRiC/CCT α resulted in G_2 mitotic arrest [134]. Cdc20 is responsible for Anaphase promoting complex or cyclosome (APC/C) activation during metaphase to anaphase transition. Cdh1 is important for maintaining the APC/C during exit from mitosis into G_1. Both Cdc20 and Cdh1 belong to a class of proteins referred as WD repeat proteins [a conserved 40 amino acid core ending with tryptophan-aspartic acid (WD)] [135], which use TRiC/CCT for protein folding. Cdc20 and Cdh1 are TRiC/CCT substrates [136]. Subsequently, upon folding, Cdc20 and Cdh1 may utilize TRiC/CCT as a platform to further assemble into a functional complex with APC/C [120]. The von Hippel-Lindau (VHL) tumor suppressor protein involved in renal clear cell carcinoma is a similar example of TriC/CCT substrate, which upon folding utilizes the chaperonin as a platform for assembly into a functional complex with elongin B and elongin C [137, 138].

1.8.1.5 Viral proteins as substrates of TRiC/CCT Chaperonin complex

Multiple viral proteins use the TRiC/CCT complex. Rabies nucleoprotein N and phosphoprotein P recruit the chaperonin γ subunit to Negri bodies (inclusion bodies) in mouse neuronal cells. Knockdown of the TRiC/CCT γ subunit results in reduced Rabies virus replication level [139]. Hepatitis C virus (HCV) non-structural protein NS5B, the viral RNA polymerase, interacts with the TRiC/CCT ε (CCT 5) subunit. Upon silencing of TRiC/CCT ε, a decrease in viral RNA is observed. The suggested mechanism is that TRiC/CCT potentially is recruited to the replication complex to aid in folding the components involved [140]. Hepatitis B virus capsid protein, a homomultimer, associates with TRiC/CCT 1 protein in the form of an assembly intermediate in a cell-free system. This interaction does not occur with the initial unassembled polypeptide or the ultimate capsid product [141]. The Gag polyprotein of Mason-Pfizer monkey virus (M-PMV) a type D retrovirus, associates with TRiC/CCT γ subunit in an ATP-dependent manner, suggesting that the chaperonin complex is involved in capsid assembly [142]. Recent reports also show that Reovirus capsid protein σ3 utilizes the TRiC/CCT complex for its assembly. The chaperonin may retain aggregation-prone regions of the σ3 protein, similar to the VHL tumor suppressor protein discussed earlier, preparing it for assembly [143]. Interestingly, a previous example of a TRiC/CCT interaction has been reported for the herpesvirus family. EBV nuclear protein 3 (EBNA-3) interacts with the ε subunit of the eukaryotic chaperonin complex [144], but the significance of this interaction remains uncharacterized.

The above examples suggest that diverse proteins, including viral proteins, fold and assemble via the TRiC/CCT complex. EBV protein provides precedence for the interaction

of eukaryotic chaperonins with herpesvirus proteins but also warrants further investigation into their role in virus biology.

1.8.2 CCR4-NOT Transcription complex

1.8.2.1 Composition of the CCR4-NOT complex

Carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) or CCR4-NOT transcription complex is a large complex with eight core subunits in mammalian cells and nine subunits in yeast. The core CCR4-NOT complex is highly conserved across eukaryotes [145, 146] and is present in flies, worms, yeast, mice, humans and plants. The yeast was utilized extensively to characterize the complex initially; however, in recent years more work has focused on mammalian cells. Yeast core complex has five NOT subunits and two catalytic modules Caf1 (Pop2) and CCR4. The human complex consists of CNOT1, CNOT2, CNOT3, CNOT4, CNOT6/6L, and CNOT7/8 and CNOT9 subunits (summarized in Table 1.4) [147, 148]. Some additional subunits identified in the human CCR4-NOT complex via co-immunoprecipitation and mass spectrometry approaches include CNOT10, CNOT11 (C2ORF29) and the 182-kDa Tankyrase binding protein (TAB182) [147, 149, 150].

The association for some of the additional components varies. For example, association of CNOT4 has been suggested to be a regulated event. TAB182 does not co-immunoprecipitate under all conditions with the CCR4-NOT complex ([147, 149, 150]. Differences in the composition of the complex also have been suggested from variation in the deadenylase subunits (CNOT6, CNOT6L, CNOT7 and CNOT8) associated with the complex [151]. Additionally, there is evidence for tissue specific expression of different subunits [152]. The multiple subunits of the CCR4-NOT complex exhibit diverse

subcellular localization and are present in both the nucleus and cytoplasm (summarized in Fig. 1.5). TAB182 is often reported to be abundantly co-immunoprecipitated with the CCR4-NOT complex [149], and yet suggested to be associated in certain conditions [150]. For this reason, I have reviewed TAB182 as a separate sub-section (1.8.2.3).

Sc (Yeast)	Mm (Mouse)	Hs (Human)	Protein Domains
Not1	CNOT1	CNOT1	
Not2	CNOT2	CNOT2	
Not3	CNOT3	CNOT3	
Not4	CNOT4	CNOT4	RING E3 Ligase
Not5			
Ccr4	CCR4	CNOT6	EEP, LRR
	CCR4L/CNOT6L	CNOT6L	EEP, LRR
Pop2/Caf1	CNOT7	CNOT7	DEDD
	CNOT8	CNOT8	DEDD
Caf40		CNOT9	
Caf130		CNOT10	
Dhh1	Rck/p54/DDX6	Rck/p54/DDX6	DEAD-box

Table 1.4: Homologues and Orthologues of the CCR4-NOT complex.

The above table summarizes orthologues of CCR4-NOT complex subunits across *Sacccharomyces cerevisiae* (Sc), *Mus musculus* (Mm) and *Homo sapiens* (Hs). Protein domains present in subunits like exonuclease-endonuclease-phosphatase (EEP), Leucine rich repeat (LRR), Asp-Glu-Asp-Asp (DEDD) and Asp-Glu-Ala-Asp (DEAD) box helicase family are shown (Adapted from Jason Miller *et al.*, 2012 [148]).

1. Yeast Not5 has no clear homologue in Ms and Hs but there is similarity with CNOT3 module in Mm and Hs.
2. Yeast CCr4 has diverged to CNOT6 and CNOT6L.
3. Yeast Caf1 has diverged to CNOT7 and CNOT8.

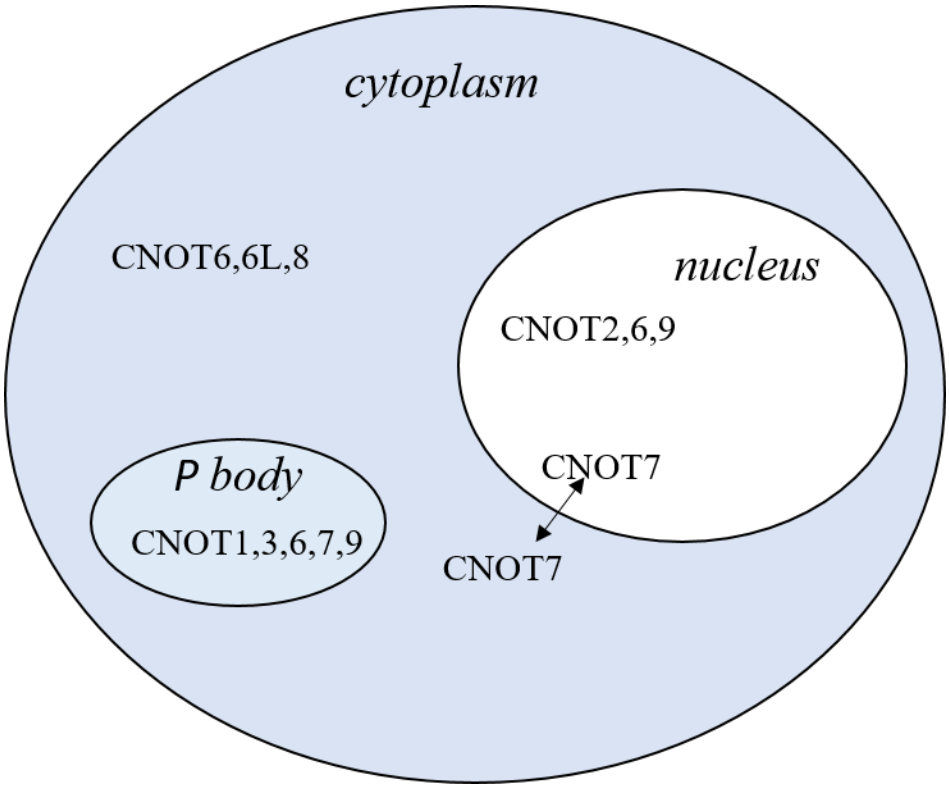


Figure 1.5: Subcellular localization of components of CCR4-NOT transcription complex in metazoans.

Human (Hs) CNOT2 and Murine (Mm) CNOT9 are mainly localized in the nucleus. Hs CNOT6, Hs CNOT6L and Mm CNOT8 are primarily present in the cytoplasm. CNOT1, 3, 6, 7 and 9 are present in P-bodies (cytoplasmic structures known to contain translationally repressed factors and mRNAs). CNOT7 is present in the nucleus and cytoplasm, depending on the stage of cell cycle (Adapted from Lau N. C. 2010 [149]).

1.8.2.2 Diverse roles of the CCR4-NOT complex

The CCR4-NOT transcription complex plays an important role in multiple processes (summarized in Fig. 1.5), and has been reviewed extensively [153-155] especially from the context of yeast system. Here I will focus on roles in mammalian cells particularly for some of the CNOT subunits relevant to this dissertation. Cell proliferation, transcriptional regulation, protein modification and deadenylation (removal of polyA residues) of mRNAs are some well-studied functions of this complex.

CNOT1 is the largest subunit and is involved in structural assembly of this complex [154]. This subunit has no catalytic activity by itself but its depletion negatively affects the deadenylase activity in HeLa cells and reduces the number of processing bodies or P-bodies (cytoplasmic structures involved in mRNA turnover) [156]. Additionally, CNOT1 interacts with specific RNA-binding proteins and determines the mRNA target specificity for deadenylase activity. This is exemplified by Tristetraprolin, an RNA-binding protein which reduces gene expression of AU-rich target mRNAs by interacting with the CNOT1 [157]. In addition, Nanos 1-3 paralogs are RNA-binding proteins, which utilize CNOT1 interaction to diminish target mRNA expression [158].

CNOT2 and CNOT3 have a conserved NOT-box at their carboxyl terminus and in this sense are structurally similar [159]. They do not possess enzymatic activities, but potentially contribute to deadenylase activity since CNOT2 depletion reduces deadenylase activity in cultured human cells [156]. CNOT2 regulates breast cancer metastasis in a mouse xenograft model [160] and is a negative regulator of Atg5-dependent autophagy [161].

CNOT4 possess RING-finger ubiquitin E3 ligase activity and showed *in vitro* ubiquitination activity. This subunit has been implicated in posttranslational regulation and protein turnover functions mediated via interaction with several accessory proteins [162].

CNOT6, CNOT6L, CNOT7 and CNOT8 are associated with deadenylase activities. Both human and mouse CNOT6 and CNOT6L possess an amino-terminal leucine-rich repeat (LRR) domain and a carboxyl-terminal endonuclease-exonuclease-phosphatase (EEP) domain [163]. Human CNOT6 and CNOT6L are paralogs and mutually exclusive in co-immunoprecipitation experiments using HeLa cells, suggesting that they have redundant functions [151]. CNOT6 is expressed in spleen, thymus, ovary and testis. In contrast, CNOT6L is ubiquitously expressed in different tissues [152]. Human CNOT6L contributes to cell proliferation by decreasing p27/kip mRNA level in MCF7 cells [164]. Additionally CNOT6L, and not CNOT6, depletion contributes to cell proliferation in NIH3T3 cells [165].

Knockout mice are generally embryonically lethal for distinct subunits of this complex. CNOT1, CNOT3, CNOT9 and CNOT10 knockout mice have an embryonically lethal phenotype (unpublished account in [154]), suggesting roles in embryogenesis. CNOT 7 knockout mice are deficient in spermatogenesis [166] and exhibit a high bone mass phenotype[167]. Together these data suggest that the CCR4-NOT complex has a multi-functional and vital role in eukaryotic cells.

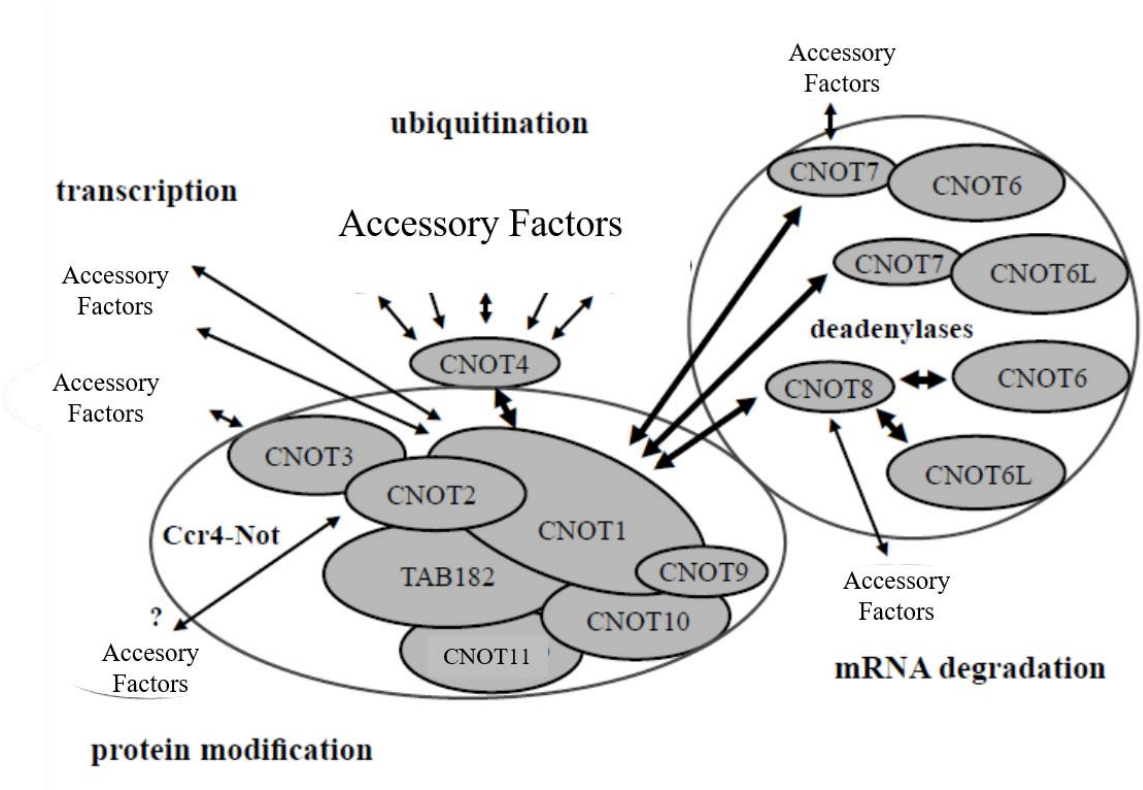


Figure 1.6: Diverse roles of the CCR4-NOT transcription complex.

The above figure summarizes role of components of CCR4-NOT complex in mRNA degradation, protein modification, transcriptional regulation (Adapted from Lau N. C., 2010 [149])

1.8.2.3 TAB182

The 182-kDa Tankyrase binding protein (TAB182) or Tankyrase 1 binding protein 1 (TNKS1BP1) was first identified in a yeast-two hybrid screen as an interacting partner and novel acceptor of Tankyrase 1 [168]. Tankyrase 1 or PARP5a is a member of the poly adenosine diphosphate (ADP) polymerase (PARP) superfamily and can modify proteins post-translationally by addition of single ADP molecules or their polymers [169, 170]. Tankyrase 1, which has a complex subcellular localization pattern and is present in both nucleus and cytoplasm, modifies TAB182 *in vitro* [168]. However, little is known about its biology.

A large-scale screen found that TAB182 is highly phosphorylated upon exposure to ionizing radiation [171]. TAB182 facilitates DNA double-strand break repair by aiding DNA-dependent protein kinase catalytic subunit (DNA-PKc) autophosphorylation in a PARP 1 dependent manner. DNA dependent protein kinase complex is an important component of non- homologous end joining (NHEJ) pathway [172]. TAB182 expression is elevated in lung adenocarcinoma tissue. Depletion of TAB182 in lung cancer cells affected its sensitivity to multiple DNA damage agents. In addition, disruption of TAB182 affected the homologous recombination (HR) pathway as evidenced by disruption of RAD51 foci formation [173]. Thus, the available reports suggest a role for TAB182 in the DNA damage response, though the mechanism is not clear. TAB182 additionally contributes to actin cytoskeleton rearrangement and cancer cell invasion [174].

In the context of the CCR4-NOT transcription complex, initially TAB182 was suggested as a species-specific component of the human complex based on co-immunoprecipitation experiments. TAB182 was not associated with the yeast complex [151]. Subsequently, numerous large-scale human protein interactome screens identified

association of TAB182 with CCR4-NOT complex subunits [175-177]. Although an earlier report suggested that TAB182 associates with CCR4-NOT complex only in certain conditions [150], a recent report argued that TAB182 is an integral component of the CCR4-NOT complex in human cells. CNOT4 was not detected in TAB182 co-immunoprecipitates, but CNOT 7 and CNOT8 were observed sporadically [178]. Thus TAB182 association with the CCR4-NOT complex might be variable depending on whether other CCR4-NOT complex subunits are associated. Tankyrase-1 interaction with TAB182, as a member of CCR4-NOT complex, is unknown. In addition, the function of TAB182 in the context of association with CCR4-NOT complex can be described best as emerging. Hence, the association of TAB182 as a component of the CCR4-NOT complex and its biology must be evaluated in a very specific context.

1.8.2.4 Interplay between viruses and components of CCR4-NOT transcription complex

Limited examples of viruses associated with constituents of CCR4-NOT complex and TAB182 have been described. Human Papillomavirus, a small DNA tumor virus, encodes the E6 protein, which is a major driver of tumorigenesis. A protein interaction screen (immunoprecipitation followed by LC-MS/MS) designed to identify protein-protein interactions of 16 different E6 proteins with host factors was able to identify CNOT1, CNOT2, CNOT3, CNOT4, CNOT6L, CNOT7, CNOT9, CNOT10, CNOT11 and TAB182 as high confidence host candidates interacting with HPV17a and HPV38 E6 proteins. Subsequently, this report validated E6 protein from HPV17a and HPV38 co-immunoprecipitates with CNOT1, CNOT2 and CNOT3 subunits. Downstream effects of this interaction are not well understood [179]. A genome-wide siRNA screen identified CNOT1, CNOT2, CNOT3 and CNOT6L subunits required for Hepatitis C virus

(*Flaviviridae*; RNA virus) replication and its mechanistic significance awaits further investigation [180]. In an attempt to identify E3 ligases contributing to Influenza A virus (IAV) replication, CNOT4, a RING E3 ligase, was identified as a host factor. Knockdown of CNOT4 diminished IAV replication. CNOT4 ubiquitinates IAV nucleoprotein (NP) and enhances viral replication. NP is the most abundant IAV protein and participates in viral RNA transcription and replication. Ubiquitination of NP CNOT4 does not lead to degradation of IAV nucleoprotein, suggesting that protein modifications contribute to the structure and function of nucleoprotein [181]. TAB182 along with CNOT3 and CNOT7 is degraded upon adenovirus serotype 5 and 12 infection. Degradation is dependent on adenovirus E1B55K and E4orf6 proteins. Depletion of TAB182 and CNOT1 promotes adenovirus replication via increased expression of E1A mRNA expression. Subsequent mechanisms need to be investigated further but suggests that TAB182 and CNOT1 control adenovirus replication [178]. Components of CCR4-NOT are thus involved in diverse ways with viral infection.

1.9 SUMMARY: OUTLINE OF THIS DISSERTATION

Through the introductory **Chapter 1**, I have highlighted the diversity of the family of herpesviruses and disease associations, the significance of HCMV mortality and morbidity, and the role of multiple small animal models to investigate HCMV. Owing to advantages associated with MCMV, I have used mice as my small animal model of choice during my dissertation research to investigate M72, a core beta herpesvirus gene. This gene was designated as a dUTPase homolog, which has examples in other members of the herpesvirus family with diverse functions. Little is known about the dUTPase homologs in the beta herpesvirus subfamily. I showed that MCMV M72 is non-functional as a dUTPase.

In **Chapter 2**, I demonstrated M72's contribution to virus replication in cell culture and the natural host.

To begin to understand the function of M72, I identified TRiC/CCT chaperonin complex and components of the CCR4-NOT transcription complex as interacting partners. In **Chapter 1,** I reviewed these host factors, novel for herpesviruses. In **Chapter 2**, I elaborated on the nature of MCMV M72 and TRiC/CCT interaction. In **Chapter 3**, I focused on the interaction and implication of components of CCR4-NOT transcription complex on MCMV M72. In **Chapter** 4, I described my overall conclusions, future avenues to explore and implications of my research.

Chapter 2: Murine cytomegalovirus M72 promotes acute virus replication *in vivo* and is a substrate of the TRiC/CCT complex[2]

2.1 INTRODUCTION

Human cytomegalovirus (HCMV), a betaherpesvirus, is the major infectious cause of birth defects in developed countries [182]. It has the potential to cause permanent neurological damage including microcephaly, cognitive impairment and sensorineural hearing loss in CMV-infected newborns [183]. Owing to the high cost of caring for children with congenital infections, HCMV has been prioritized as a candidate for vaccine development in the United States [184]. Immunocompromised individuals, including transplant recipients and HIV-infected persons, represent another susceptible population in which HCMV represents a major cause of morbidity and mortality. In this group, problems arise due to acute infection or reactivation, including retinitis, hepatitis, and pneumonitis [185, 186].

The narrow host range of HCMV presents a challenging for study of the role of viral genes in the context of the natural host. Hence, MCMV infections of mice are used as a tractable animal model for studies of CMV pathogenesis. Sequence and functional homologs of viral genes among the CMVs of different hosts facilitate studies of specific pathogenic mechanisms [187, 188]. MCMV has a wide variety of genes that contribute to

[2]Large portions of this chapter are part of the paper [1]. Ibid..
Briefly, following authors contributed to the paper – Sandhya Gopal (SG), Encarnacion Perez Jr. (EP), Amanda Y. Xia (AX), Jonathan J. Knowlton (JK), Filipe Cerqueira (FC), Terence S. Dermody (TD) and Jason W. Upton (JWU). SG and JWU designed the experiments. SG, EP, AX, JK, FC, JWU performed the experiments. SG wrote the manuscript and primarily JWU edited it. TD, JK, EP and FC provided additional input in manuscript writing.

its ability to infect and evade the host responses. This leads to the establishment of an intricate life-long host pathogen relationship, characteristic of all herpesviruses. The gamut of MCMV genes includes a subset of core genes that are evolutionarily conserved across herpesviruses. Most of these genes encode proteins required for replication and virus structure. However, many core genes remain relatively uncharacterized, and little is known about their contribution to infection. The MCMV gene, M72, designated as a 2' deoxyuridine 5'triphosphate pyrophosphatase (dUTPase) homolog, is one such example of a core gene with no identified function [189].

Cellular dUTPases are ubiquitous enzymes that convert dUTP to dUMP and pyrophosphate (PPi) to control cellular nucleotide pools and prevent misincorporation of uracil into cellular DNA. There are several examples of viral dUTPases among retroviruses and DNA viruses [94]. The dUTPase-encoding gene in non-primate lentiviruses is essential for replication in non-dividing cells [98]. Similarly, a feline immunodeficiency virus (FIV) dUTPase mutant displays a reduced viral burden *in vivo*, suggesting a contribution to infection in the natural host [99]. Among herpesviruses, a dUTPase-encoding gene is present in all three herpesvirus family subdivisions, and the alpha- and gamma-herpesviruses dUTPases are functional enzymes [100]. The varicella zoster virus (VZV) and simian varicella virus (SVV) dUTPases, encoded by ORF8 of each virus, contribute to virus replication in cell culture [104, 190]. Additionally, a null mutant for the HSV 1 dUTPase, UL50, is less virulent compared to wild type virus and exhibits decreased neurovirulence [102].

Many herpesvirus dUTPases have functions independent of their dUTPase activity. KSHV and murid herpesvirus-68 (MHV-68) ORF54 down-regulate a cell-surface ligand, NKp44L [109], and degrade IFN receptor 1 protein [110], respectively. EBV dUTPase, BLLF3, activates NF-κB [107] and induces the secretion of pro-inflammatory cytokines in a TLR2-dependent manner [108]. Thus, some are functions associated with alpha- and gammaherpesvirus designated dUTPases are independent of their catalytic activity.

Little is known about the beta herpesvirus dUTPase homologs. Preliminary characterization of the HCMV designated dUTPase homolog, UL72, revealed that this protein is catalytically inactive and dispensable for replication in cell culture [191]. Here, I report that the MCMV designated dUTPase homolog, M72, is also non-functional as a dUTPase enzyme, augments virus replication in some cell types, and contributes to viral pathogenesis in the acute phase of replication in the natural host. The M72 protein is expressed from early times post infection, shows a complex expression profile that includes multiple shorter isoforms, and is a substrate of the eukaryotic chaperonin tailless complex protein -1 (TCP-1) ring complex (TRiC)/chaperonin containing tailless complex polypeptide 1 (CCT). Together, this initial characterization of M72 reveals new insight into betaherpesvirus dUTPase homologs and highlights the contribution of M72 to viral pathogenesis.

2.2 RESULTS

2.2.1 M72 is not an active dUTPase

MCMV M72 is designated as a dUTPase homolog based on limited sequence similarity with other herpesviruses and homology to HCMV UL72 [13]. To determine

whether M72 possesses intrinsic dUTPase activity, a previously described approach was employed [110]. Briefly, transfected viral dUTPases and homologs were purified from cell lysates and incubated with dUTP, where functional dUTPase enzymes convert dUTP to dUMP and pyrophosphate. The sample was then used in place of dTTP in PCR, where dUTP that has not been enzymatically processed will be incorporated into the amplicon and result in a successful reaction. MHV-68 ORF54 and its catalytic mutant, ORF54$^{H80A/D85N}$ [110], were used as positive and negative controls, respectively. Immunoblots confirmed expression and immunoprecipitation of transfected, epitope tagged M72 and ORF54 proteins (Fig. 2.1A). As expected, MHV-68 ORF54 showed enzymatic activity as a dUTPase, consuming dUTP in the *in vitro* reaction. The ORF54$^{H80A/D85N}$ catalytic mutant was incapable of depleting provided dUTP, resulting in a positive PCR (Fig. 2.1B). In comparison to the controls, both amino- and carboxy-terminal epitope tagged M72 did not display dUTPase activity similar to the MHV68 ORF54 catalytic mutant (Fig. 2.1B). These results suggest that the MCMV M72 gene product is not an active dUTPase enzyme, likely playing an alternative role in virus replication.

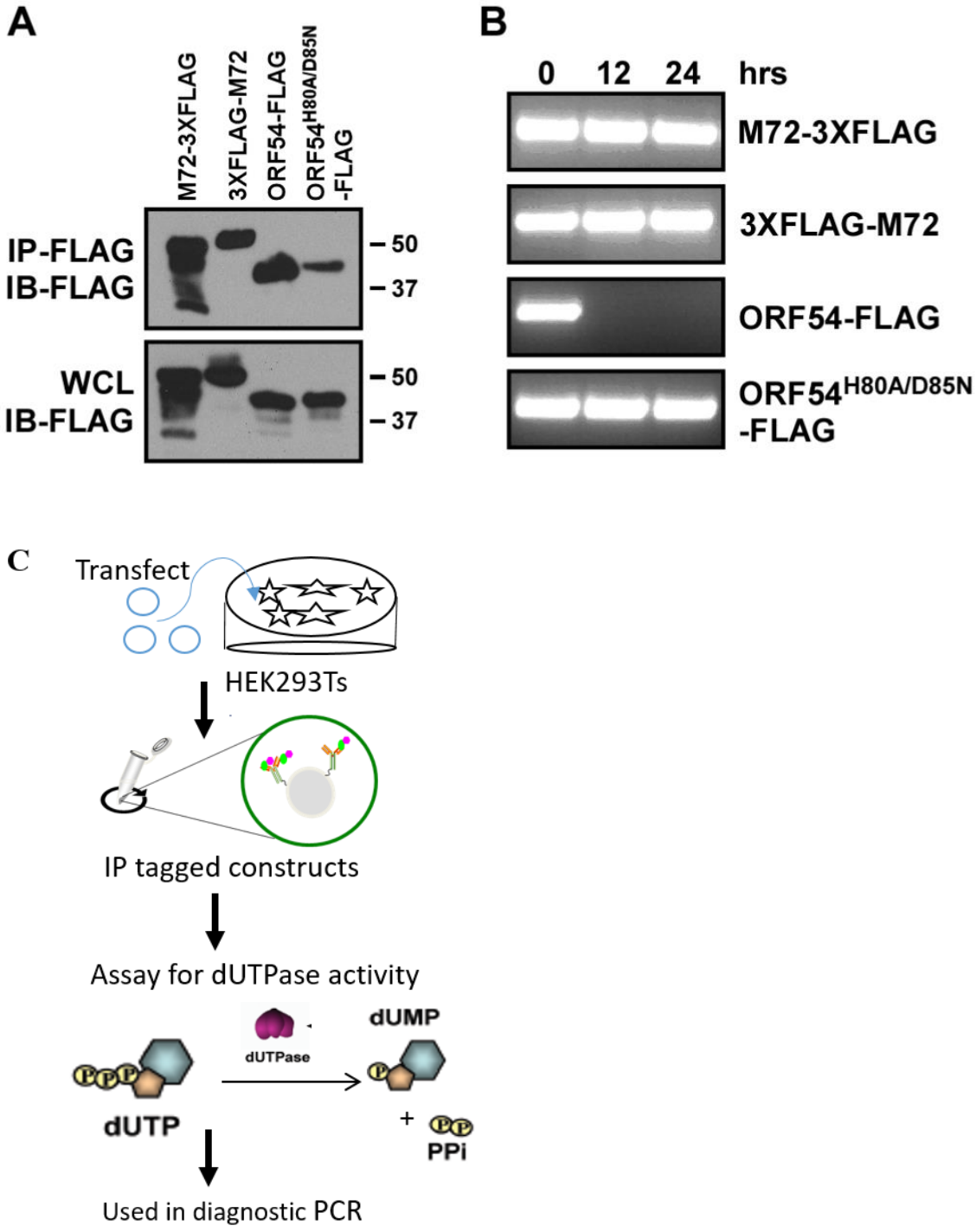

A
M72-3XFLAG
3XFLAG-M72
ORF54-FLAG
ORF54H80A/D85N -FLAG
IP-FLAG IB-FLAG
WCL IB-FLAG
50
37
50
37
B
0 12 24 hrs
M72-3XFLAG
3XFLAG-M72
ORF54-FLAG
ORF54H80A/D85N -FLAG
C
Transfect
HEK293Ts
IP tagged constructs
Assay for dUTPase activity
dUTPase
dUMP
dUTP
+
PPi
Used in diagnostic PCR

Figure 2.1: M72 is not an active dUTPase.

A) Immunoblot (IB) analysis of immunoprecipitations (IP) and whole cell lysates (WCL) from HEK293T cells transfected 24 hr with the indicated expression vector. B) Ethidium bromide (EtBr) stained agarose gel of PCR utilizing individual dNTPs, with dTTP replaced by dUTP following incubation with IPs from (A). Presence of a PCR product indicates intact dUTP, and a non-functional dUTPase homolog. C) Outline of the assay.

2.2.2 Generation of M72 mutant viruses

To investigate the potential role of M72 in MCMV pathogenesis, the pARK25 bacterial artificial chromosome (BAC) containing the K181 (Perth) strain of MCMV [192] was used to introduce two independent premature stop codons into the M72 open reading frame with established recombineering techniques (see in Fig. 2.2A) [192]. Initially, a selection/counter selection cassette (sacB/Kan^R) was inserted into a region including the M72 open reading frame (corresponding to the MCMV genome between nucleotide 102,772–105,791) by allelic exchange. This cassette was replaced in a second allelic exchange step with two independent amplicons containing engineered stop codons inserted within the M72 gene. The 5'-end of the M72 gene on the complementary strand was overlapped by the 5' ends of M73 and M73.5 genes. To prevent disruption of the M73 and M73.5, mutations were inserted into a non-overlapping region in the 5' end of M72. The BACs were analyzed by restriction fragment length polymorphism (RFLP) and PCR/restriction digest to ensure the structural integrity and presence of intended mutations. Isolated BAC DNA digested with *HindIII* or *PstI* revealed the anticipated patterns due to the insertion of the sacB/Kan^R cassette. The M72.SK BAC digested with *HindIII* showed the appearance of a unique 2,007 bp band, whereas PstI digestion revealed the loss of a 4,931 bp band and addition of a unique 8,828 bp band. These differences were absent from the specific mutant BACs, which were indistinguishable from WT BAC (Fig. 2.2B). As expected, amplification of the region around M72 from WT and mutant BACs produced amplicons of 2,082 bp, whereas the sacB/Kan^R containing M72.SK produced an amplicon of 4,000bp. Moreover, digestion of amplicons with *SpeI* or *NheI* revealed the insertion of

specific mutations linked to each unique diagnostic restriction enzyme site in M72StopS and M72StopN (Fig. 2.2C), respectively. WT and mutant BAC DNAs were transfected into NIH3T3 fibroblasts and viruses were recovered and amplified. No major differences in viral immediate-early (m123/IE1), early (m112-113/E1) or late (MCP/M86) proteins were seen in NIH3T3 cells infected with WT, M72StopS or M72StopN (Fig. 2.2D), suggesting that M72 likely does not influence viral protein expression during infection.

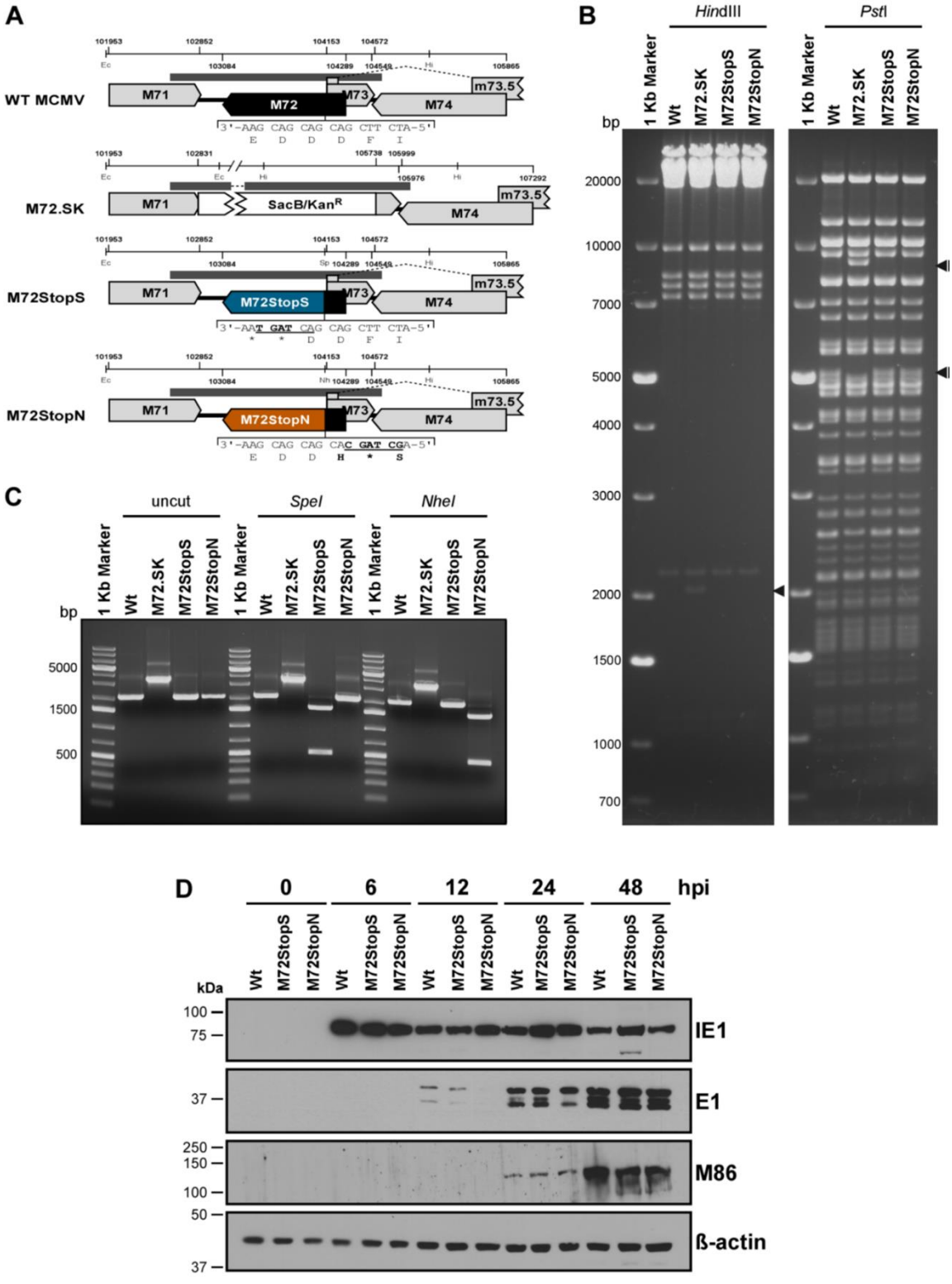

A
WT MCMV
M72.SK
M72StopS
M72StopN
M71
M72
M73
M74
m73.5
SacB/Kan^R
M72StopS
M72StopN
B
HindIII
PstI
1 Kb Marker
Wt
M72.SK
M72StopS
M72StopN
bp
20000
10000
7000
5000
4000
3000
2000
1500
1000
700
C
uncut
SpeI
NheI
bp
5000
1500
500
D
0
6
12
24
48
hpi
kDa
100
75
37
250
150
100
50
37
IE1
E1
M86
ß-actin

Figure 2.2: Generation of M72StopS and M72StopN recombinant viruses.

A) Schematic diagram of genomic location of M72 and mutagenesis strategy to generate M72StopS and M72StopN. Numbers represent MCMV genomic coordinates (GenBank Accession number AM886412.1), and abbreviations indicate restriction enzyme sites (Hi, HindIII; Ec, EcoRI; Sp, SpeI; Nh, NheI). M72StopS and M72StopN were generated by a two-step allelic exchange strategy (see Materials & Methods) inserting, and then replacing, a selection/counterselection cassette (SacB/KanR). Nucleotide and amino acids changed are indicated in bold, and underlined sequences represent introduced diagnostic restriction enzyme sites. Grey bars represent diagnostic PCR amplicons in C). B) RFLP analysis of WT, M72.SK, M72StopS and M72StopN BACs. Isolated DNA was digested with the indicated enzyme, separated on a 0.6% agarose gel and visualized by EtBr staining. Arrowheads indicate specific important DNA fragments addressed in Results. C) Infectious virion DNA from the indicated viruses was isolated, and amplicons from the M72 locus generated by PCR. Amplicons were digested with the indicated enzyme or left uncut. Products were separated on a 1.0% agarose gel and visualized by EtBr staining. D) Western blots of MCMV IE1, E1, M86 and β-actin expression from lysates of NIH3T3 cells infected 0-48 hr with the indicated virus (MOI=5.0 PFU/cell).

2.2.3 MCMV M72 augments viral replication in cell culture.

To begin to understand the contribution of M72 to viral replication, the replication of M72StopS and M72StopN mutants were characterized in cell culture. In addition to murine fibroblasts, I assessed viral growth in endothelial and macrophage cell types, both of which are important for CMV infection. Analysis of yields during single-step infection (MOI = 5.0 PFU/cell) showed that M72StopS and M72StopN mutants were attenuated by nearly 10-fold in murine fibroblasts (Fig. 2.3A) and endothelial cells (Fig. 2.3B) compared with WT virus. However, both the M72Stop mutant viruses replicated to comparable levels as the WT virus in a macrophage cell line (Fig. 2.3C) and in bone marrow derived macrophage (BMDM) primary cells (Fig. 2.3 D). Multi-step infection (MOI = 0.05 PFU/cell) revealed similar trends in each of the three cell types; mutant viruses produced lower levels than WT virus in NIH3T3 and SVEC4-10 cells (Fig. 2.3E,F) and similar levels as WT virus in the macrophage cell line (Fig. 2.3G). Together, these results indicate that M72 augments virus replication in specific types of cultured cells.

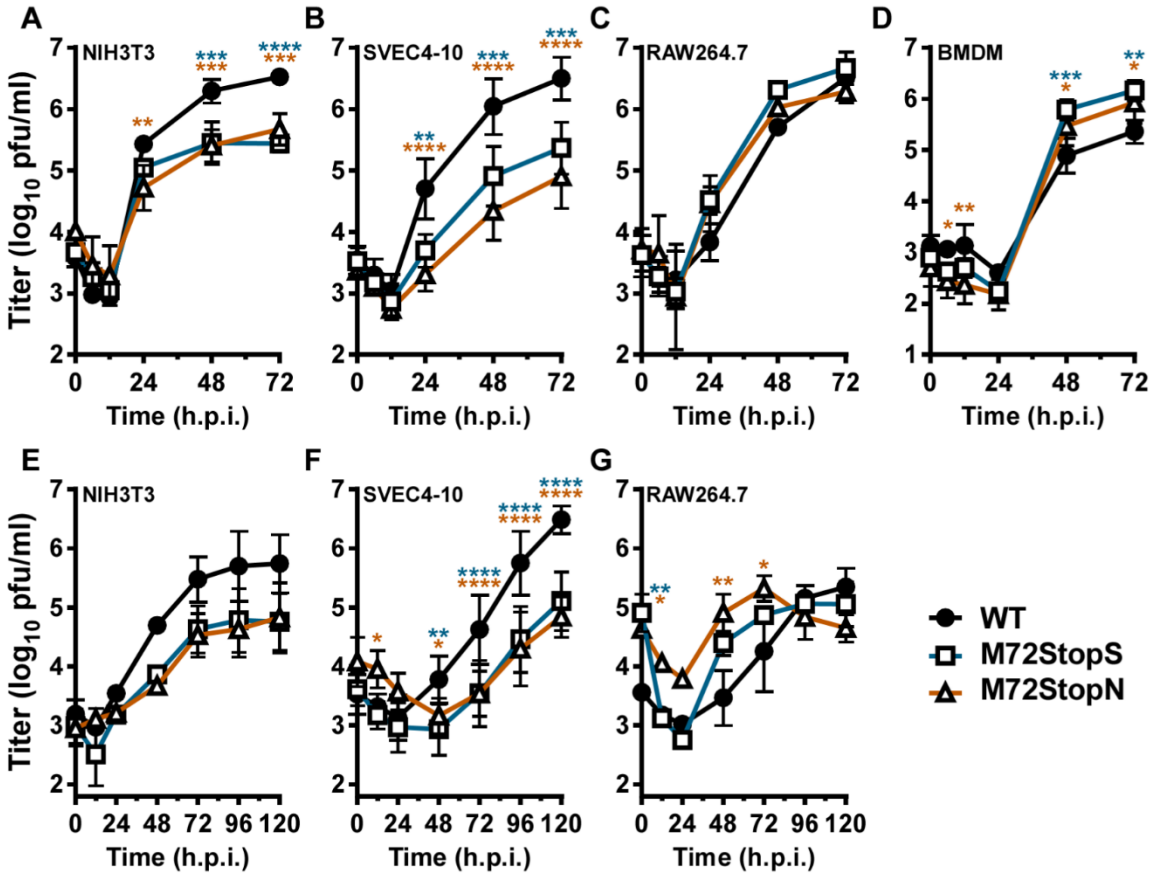


Figure 2.3: MCMV M72 augments virus replication in cell culture.

(A to D) Single-step (5.0 PFU/cell) and (E to G) Multi-step (0.05 PFU/cell) growth curves in NIH3T3 fibroblasts (A and D), SVEC4-10 endothelial cells (B and E), RAW264.7 macrophages (C and F), and BMDM primary cells (D) infected with WT, M72StopS or M72StopN recombinant viruses. Each data point represents n = 3 to 6 replicates. Error bars represent standard deviation. Statistical analysis at each time point was performed using two-way ANOVA analysis with a Tukey's multiple comparison test. * - $p<0.01$, ** - $p<0.001$, *** - $p<.0001$, **** - $P<.00001$. Color of asterisks denotes the statistical difference of each mutant from WT. The 0 hr time point represents the time immediately post wash and addition of fresh complete media. h.p.i.; hours post infection.

2.2.4 MCMV M72 augments virus replication in the early phase of acute infection in the natural host.

Since M72 plays a role in efficient replication in cell culture, I next sought to determine how disruption of M72 affects virus replication in the context of a natural host. BALB/cJ mice were infected via intraperitoneal (i.p.) inoculation with 10^5 PFU of the WT, M72StopS or M72StopN viruses, and viral loads in the spleen and salivary glands were determined. Compared with the WT virus, M72StopS and M72StopN viruses were each attenuated approximately 10-fold in spleens on day 3 post-inoculation (Fig. 2.4A). At day 5 post-inoculation, a similar trend was observed in infected spleens, although with greater variation in titers, and differences between the loads of WT and M72Stop mutants did not reach statistical significance (Fig. 2.4B). Surprisingly, analysis of salivary gland titers at day 7 (Fig. 2.4C) and day 14 (Fig. 2.4D) post-inoculation demonstrated that the M72Stop mutants were present at similar levels as the WT virus. This finding indicates that the capacity of the M72Stop mutant viruses to disseminate in the natural host remains unaffected. Together, these data suggest that MCMV M72 contributes to acute replication of the virus during the early phase of infection, but is dispensable for dissemination in the natural host. Thus, a betaherpesvirus dUTPase plays a role in viral pathogenesis.

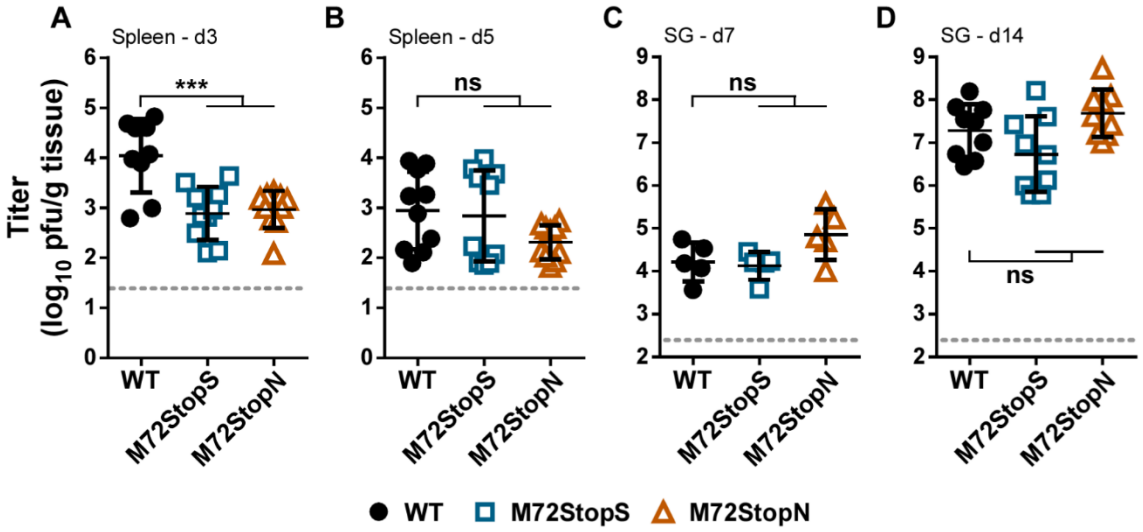


Figure 2.4: MCMV M72 augments virus replication in the early phase of acute infection in the natural host.

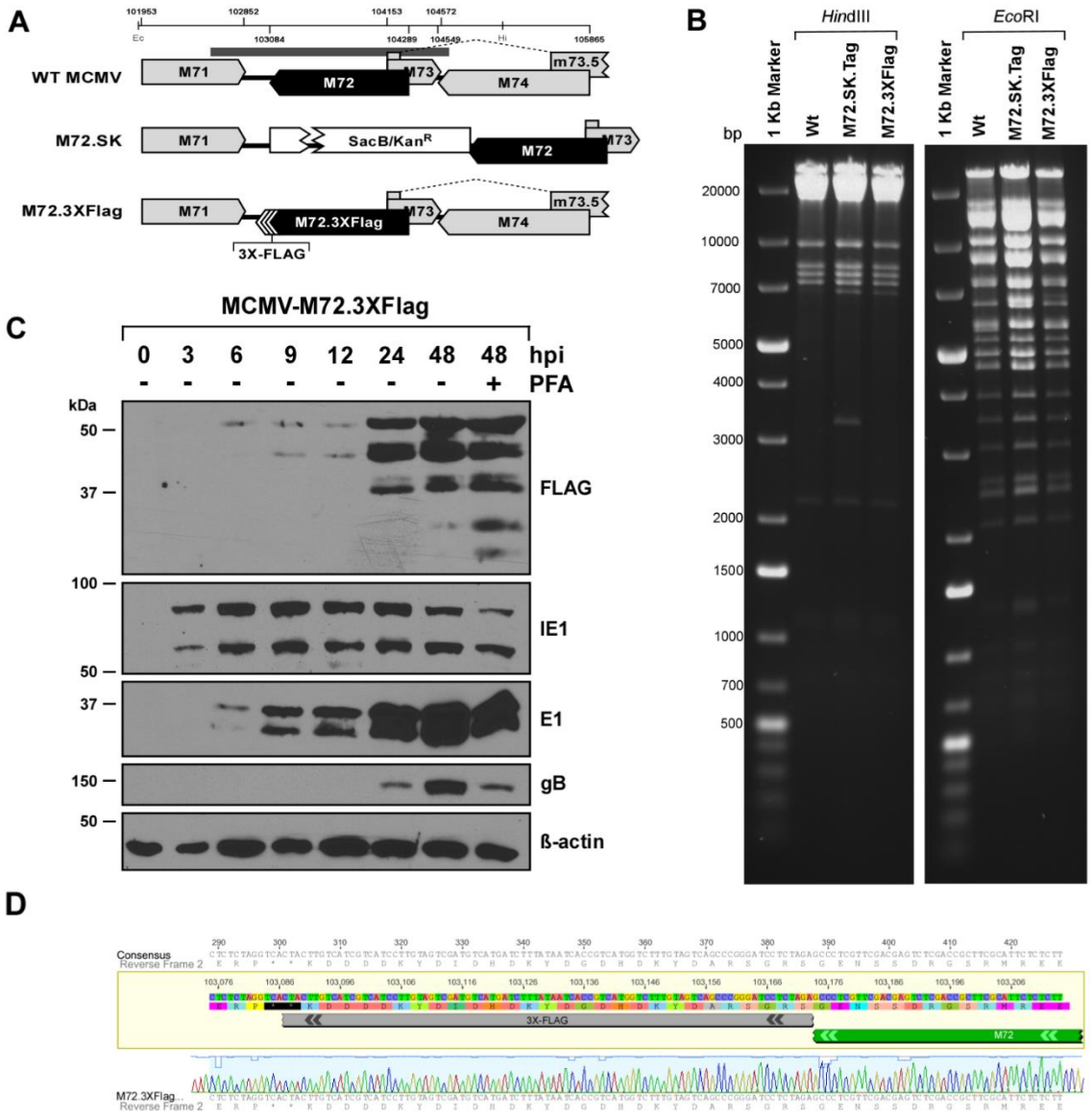
(A to D) Organ titers from BALB/cJ mice infected with 10^5 PFU of either WT, M72StopS or M72StopN viruses. Spleen (A-B) and salivary gland (C-D) were collected at the indicated day (d) post infection, and virus titer determined by plaque assay on NIH3T3 fibroblasts. Each data set for individual virus represents n = 10-15 mice per group. Error bars represent standard deviation. Statistical analysis at each time point was performed using two-way ANOVA analysis with a Tukey's multiple comparison test. *** - p<.0001, ns – not significant.

2.2.5 Generation of tagged virus and evaluation of M72 protein expression.

Although introduction of premature translational stop codons into the predicted M72 ORF affected viral replication both *in vitro* and *in vivo*, little is known about the protein expression of M72. M72 is encoded in a transcriptionally complex region of the genome, and transcripts corresponding to the M72 gene have been detected in infected cells [193-195]. Moreover, peptides corresponding to M72 sequence have been detected in MCMV virions [196]. However, the expression of M72 protein during infection has not been reported. Since M72-specific antibodies are not available, I engineered a recombinant virus in which a 3X-FLAG epitope tag was appended to the carboxyl-terminus of M72. The predicted stop codon of M72 (nucleotides 103,083–103,086) of the K181 BAC was replaced with a sacB/Kan^R cassette via recombineering. In the subsequent step, this cassette was replaced with an amplicon containing a 3X-FLAG epitope fused to the carboxyl-terminal end of M72 to generate MCMV M72.3XFlag (Fig. 2.5A). RFLP analysis (Fig. 2.5B), PCR amplification and sequencing (Fig. 2.5D) confirmed the presence of the inserted sequence, and infectious virus was recovered.

To quantify the expression of M72 during infection, NIH3T3 fibroblasts were infected with M72.3XFlag at an MOI of 5 PFU/cell. Samples were harvested at the time of virus addition (t=0) and at various intervals thereafter for immunoblot analyses. Expression of a ~46kDa band, consistent with the estimated and observed molecular mass of full length, epitope tagged M72 (Fig. 2.1A) was observed as early as 6 hours post infection (h.p.i.), reaching peak levels between 24 and 48 h.p.i. (Fig. 2.5C). Expression of immediate-early (m123/IE1), early (m112-113/E1) and late (gB/M55) gene products were observed as anticipated (Fig. 2.5C). Addition of the CMV DNA polymerase inhibitor phosphonoformic acid (PFA) at the time of infection did not interfere with the expression

of IE1 or E1, but significantly reduced the expression of the late protein gB (Fig. 2.5C). M72 has previously been designated a late gene based on RNA expression [195]. Inconsistent with this, M72 protein expression was not adversely affected by the addition of PFA, but instead showed enhanced protein expression of faster migrating isoforms of M72. Interestingly, additional, faster migrating protein bands were observed over the time course of infection. A similar pattern was observed upon detection of exogenously expressed FLAG-tagged M72 (Fig. 2.1A, 2.6B, 2.7B), suggesting that the initial observation was not an artifact and that multiple M72 protein isoforms accumulate during infection. Based on the approximate molecular masses of the faster migrating isoforms, and the number and locations of multiple methionine residues in the N-terminus of M72, we hypothesized that initiation of M72 protein isoform expression could be happening from internal methionine residues. A series of N-terminal truncations were constructed in which each methionine between M1 and M140 were used as the initiating methionine for the construct (Fig. 2.5E). Immunoblot analysis of NIH3T3 cells transfected with each of the amino-terminal truncations were compared to MCMV-M72.3XFLAG infected cells analyzed on the same gel. Virus-infected cells showed the expected pattern of multiple M72 protein isoforms (Fig. 2.5F, left panel), and bands in infected cells migrated consistent with expression constructs encoding full length M72 $^{(1\text{-}401)}$, M72 $^{(46\text{-}401)}$, M72 $^{(76\text{-}401)}$ and M72 $^{(139\text{-}401)}$ (Fig. 2.5F, right panel). Together, these results suggest a complex expression profile of M72 during infection, consistent with internal initiation on different methionine.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WT MCMV
M72.SK
M72.3XFlag
M71
M72
M73
M74
m73.5
SacB/Kan^R
M72.3XFlag
3X-FLAG
B
HindIII
EcoRI
1 Kb Marker
Wt
M72.SK.Tag
M72.3XFlag
bp
20000
10000
7000
5000
4000
3000
2000
1500
1000
700
500
C
MCMV-M72.3XFlag
0 3 6 9 12 24 48 48 hpi
- - - - - - - + PFA
kDa
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100
50
37
150
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IE1
E1
gB
ß-actin
D
Consensus
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3X-FLAG
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M72.3XFlag...
Reverse Frame 2

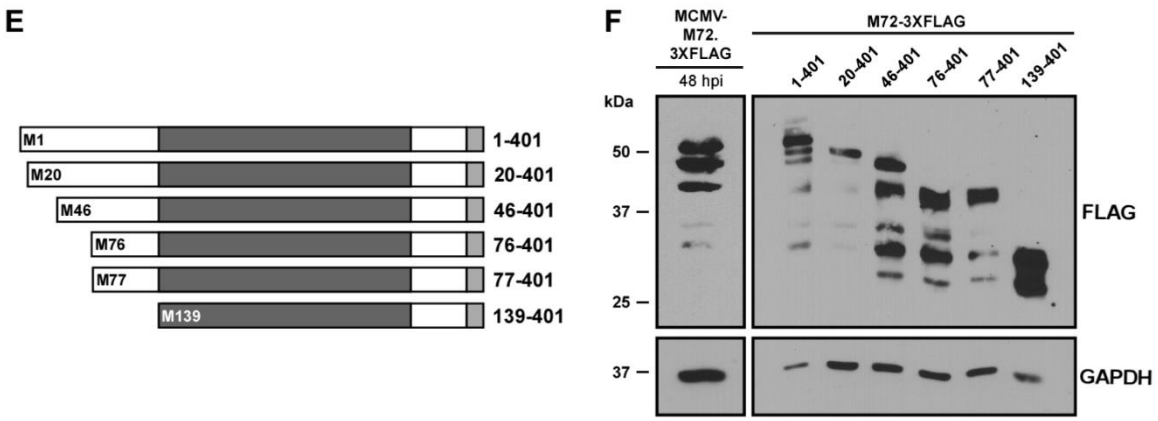


Figure 2.5: Expression of M72 protein during MCMV infection.

A) Schematic diagram of genomic location of M72 and mutagenesis strategy to generate M72.3XFlag. Numbers represent MCMV genomic coordinates (GenBank Accession number AM886412.1), and abbreviations indicate restriction enzyme sites (Hi, *HindIII*; Ec, *EcoRI*). M72.3XFlag was generated by a two-step allelic exchange strategy (see Materials & Methods) inserting, and then replacing, a selection/counterselection cassette (SacB/Kan^R) with a 3XFLAG epitope tag. B) RFLP analysis of WT, M72.SK.Tag, and M72.3XFlag BACs. Isolated DNA was digested with the indicated enzyme, separated on a 0.6% agarose gel and visualized by EtBr staining. C) Immunoblot for FLAG, IE1, E1, gB and actin from whole cell lysates of NIH3T3 cells infected with (MOI=5.0 PFU/cell) MCMV M72.3XFlag virus in the presence or absence of 200 µg/mL PFA. Samples were collected at the indicated time points post infection, separated by SDS-PAGE and western blot analysis. The 0 hr time point represents the time of addition of virus to the dishes. D) Representative results of sequencing and alignment of the M72.3XFlag insertion site. Amplicons were generated from viral genomic DNA using primers flanking the end of M72. E) Schematic diagram of epitope-tagged M72 N-terminal mutants initiating from specific methionine residues within the open reading frame. Numbers preceded by "M" denote the amino acid position of the internal methionine for initiation of protein expression for each mutant. Dark Grey box indicates putative 'dUTPase' domain. Light grey box indicates 3XFlag epitope tag. Numbers denote amino acid numbers. F) Immunoblot analysis for FLAG and GAPDH of NIH3T3 cells transfected with the indicated N-terminal FLAG-tagged M72 construct, or infected with MCMV- M72.3XFlag.

2.2.6 Identifying interacting partners of M72.

To begin to understand the function of M72, I sought to define cellular interacting protein partners of M72. M72-3XFlag or control plasmid were transfected into NIH3T3 fibroblasts. FLAG-immunoprecipitates were prepared, resolved by 10% SDS-PAGE and stained. Multiple bands were observed in the M72-3XFLAG sample relative to the vector control (Fig. 6A). A region of each lane in which multiple bands were differentially enriched, corresponding to approximately 55-70 kDa, were excised from each sample, subjected to in-gel tryptic digestion and LC-MS/MS analysis. Peptide sequences were compared to the UniProt mouse reference genome, and used to generate an initial list of 109 total unique proteins, excluding those of low confidence representation, low abundance, or those enriched in the control sample. Proteins for which a minimum of 10 peptide spectral matches in the experimental sample were detected, and those, which showed at least a 2-fold enrichment over control samples were, designated candidate interacting proteins (Table 2.1).

Among the candidates meeting these criteria, all eight subunits of the TRiC/CCT complex were significantly enriched in M72-containing immunoprecipitates. TRiC/CCT is a hetero-oligomeric complex that aids in cellular protein folding and assembly. The complex is a eukaryotic chaperonin and has eight paralogous subunits (TRiC/CCT 1-8) arranged in a stacked ring-like structure [197]. TRiC/CCT functions in an ATP-dependent manner and provides a physically defined compartment in which cellular protein domains or entire proteins can fold while being sequestered from the cytosol [198]. The TRiC/CCT complex interacts with viral proteins such as the EBNA-3 protein

of EBV [144], hepatitis C virus NS5B [140], influenza PB2 [199], rabies virus N and P proteins [139], and reovirus σ3 capsid protein [200], indicating that it is an important cellular factor across diverse groups of viruses.

Identified protein	UNIPROT Accession	kDa	Peptide spectral counts (PSM)		Fold enrichment
			Con	M72-	
T-complex protein 1 subunit epsilon (Cct5)	TCPE_MOUSE	60	2	151	75.5
T-complex protein 1 subunit eta (Cct7)	TCPH_MOUSE	60	2	150	75.0
tRNA-splicing ligase RtcB homolog	RTCB_MOUSE	55	1	39	39.0
T-complex protein 1 subunit beta (Cct2)	TCPB_MOUSE	57	4	149	37.3
T-complex protein 1 subunit zeta (Cct6a)	TCPZ_MOUSE	58	3	110	36.7
T-complex protein 1 subunit delta (Cct4)	TCPD_MOUSE	58	5	144	28.8
T-complex protein 1 subunit theta (Cct8)	TCPQ_MOUSE	60	8	174	21.8
T-complex protein 1 subunit alpha (Tcp1)	TCPA_MOUSE	61	11	142	12.9
Polymerase I and transcript release factor	PTRF_MOUSE	44	2	15	7.5
T-complex protein 1 subunit gamma (Cct3)	TCPG_MOUSE	61	0	169	-
60S ribosomal protein L4	RL4_MOUSE	61	0	38	-
CCR4-NOT transcription complex subunit 2	CNOT2_MOUSE	60	0	34	-
CCR4-NOT transcription complex subunit 6-like	CNO6L_MOUSE	63	0	18	-
Heterogeneous nuclear ribonucleoprotein K	B2M1R6_MOUSE	49	0	17	-
Protein FAM98A	FA98A_MOUSE	55	0	14	-
Nucleolin	NUCL_MOUSE	77	0	13	-
Protein disulfide-isomerase	PDIA1_MOUSE	57	0	12	-
26S protease regulatory subunit 4	PRS4_MOUSE	49	0	11	-
Isoform 3 of Myelin expression factor 2	MYEF2_MOUSE	63	0	11	-
Heterogeneous nuclear ribonucleoprotein L	G5E924_MOUSE	67	0	10	-

Table 2.1: Candidate M72-interacting cellular proteins identified by mass spectrometry.

2.2.7 M72 associates with and is a substrate of the TRiC/CCT complex.

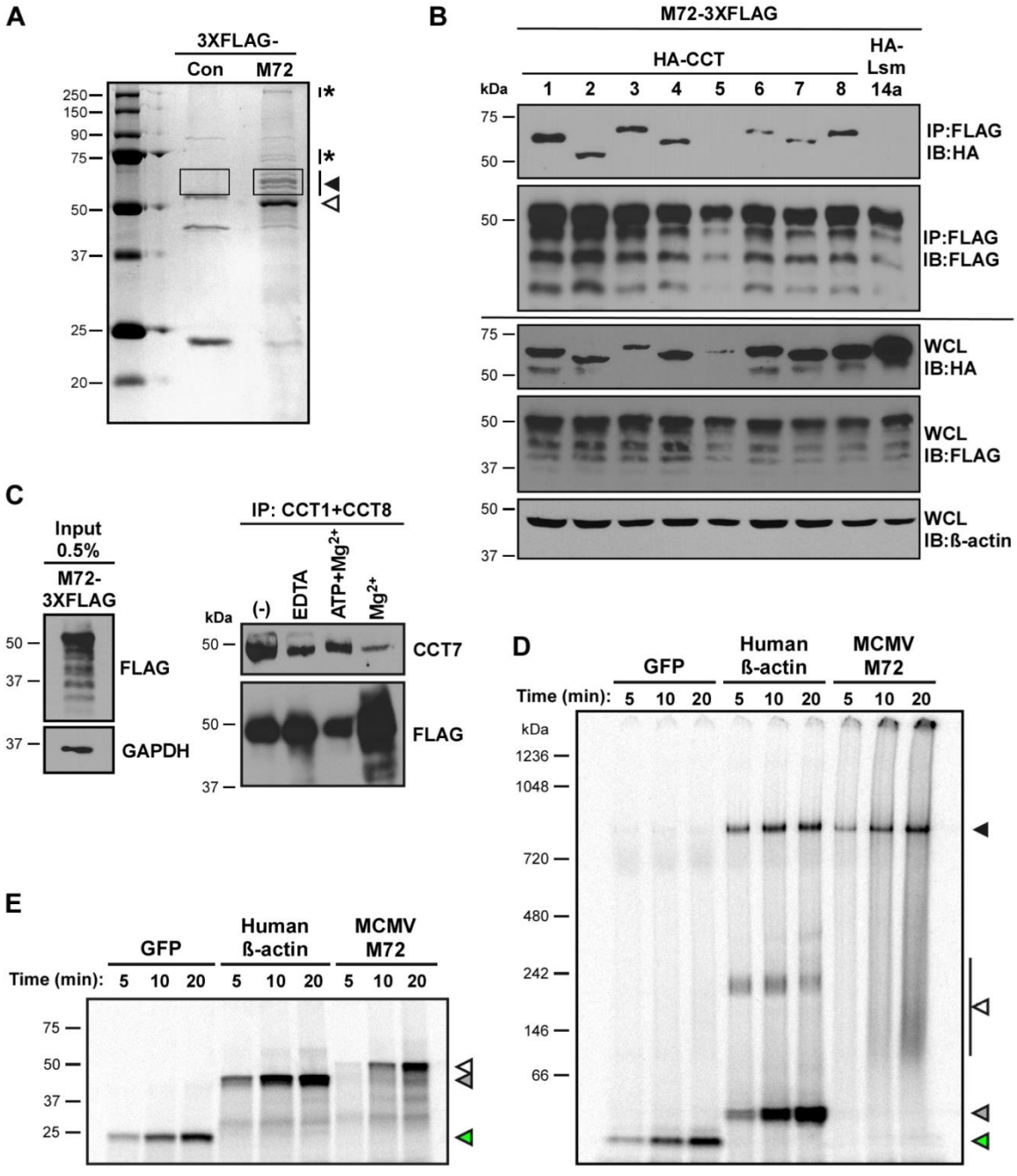
To validate the results of the MS experiment, M72-3XFLAG and each of the HA-tagged CCT 1-8 subunits were transiently co-expressed in HEK293T cells. Immunoprecipitation followed by immunoblotting revealed that M72 co-immunoprecipitated each of the co-transfected subunits of TRiC/CCT complex, except CCT5 (Fig. 2.6B). Interestingly, CCT5 was consistently and reproducibly expressed to significantly lower levels when co-transfected with M72. Whether this represents an artifact of co-expression, or is indicative of an antagonism remains unclear. Thus, I am unable to conclusively confirm the interaction between M72 and CCT5 observed by mass spectrometry (Table 1). An irrelevant, comparably sized and epitope- tagged protein, Lsm14a, did not co-immunoprecipitate with M72 indicating the interaction was specific. Similar findings were made using M72 with an epitope tag appended to the amino- (data not shown) terminus, indicating that the epitope tag did not influence the interaction.

Since the TRiC/CCT complex is an interacting partner with M72 and required for homeostatic protein folding of many cellular proteins, such as actin [201], M72 might modulate TRiC/CCT complex activity. However, infection of cells with WT, M72StopS, or M72StopN viruses showed no discernable differences or changes of expression of individual CCT proteins, and actin protein levels remained constant throughout each infection condition (data not shown). This finding suggested that M72 does not affect the expression or activity of the TRiC/CCT complex during MCMV infection and raises the possibility that M72 is a client protein of the TRiC/CCT complex.

To determine whether M72 is a substrate of the TRiC/CCT complex, I employed a CCT substrate assay [202]. TRiC/CCT substrates should be released from the complex in the presence of $MgCl_2$ and ATP, but remain associated when treated with a divalent cation chelator, such as EDTA. Thus, to assess the effect of these treatments on M72 release from isolated TRiC/CCT complex, cell lysates were prepared from M72-3XFLAG-transfected HEK293T cells, and divided into four equal aliquots. Aliquots were incubated with either EDTA, $MgCl_2$ or, $MgCl_2$ with ATP for 40 min at room temperature. Samples were then immunoprecipitated with a mixture of anti-CCT1 and CCT8 antibodies to isolate TRiC/CCT complexes and their associated substrates and then analyzed by SDS-PAGE and immunoblotting. TRiC/CCT substrates should be released in the presence of $MgCl_2$ and ATP, but remain associated with the complex when treated with a divalent cation chelator, such as EDTA. Immunoblotting for M72 revealed a substantial decrease in M72 co-precipitated with TRiC/CCT complexes in the presence of $MgCl_2$ and ATP, whereas comparable levels of M72 were observed in mock- and EDTA-treated samples (Fig. 2.6C). This result confirms the interaction of M72 with the TRiC/CCT complex and suggests that M72 is dissociated from the complex in an ATP-dependent manner.

To more directly test whether M72 is a TRiC/CCT substrate, I investigated the association of *in vitro* translated M72 with TRiC/CCT over time in rabbit reticulocyte lysates (RRLs). RRLs are a common tool used to identify and characterize TRiC/CCT substrates [203, 204], including human β-actin [201] and Reovirus σ3 [200]. Actin (an

obligate TRiC/CCT substrate), GFP (which does not require TRiC/CCT to fold) or M72 were translated *in vitro* in the presence of S^{35}-labeled methionine for 5, 10, or 20 min. Reactions were resolved on parallel native and SDS-PAGE gels. As anticipated [200], newly translated actin was observed in association with a large complex (>750kDa) in native gels, corresponding to the actin/TRiC complex [200, 201, 205]. Over the time course, a low molecular weight (<60kDa) form of actin accumulated, consistent with the release of monomeric actin from TRiC/CCT (Fig. 2.6D). GFP, which is not a TRiC/CCT substrate, did not form a high molecular weight complex with TRiC/CCT, and instead consistently accumulated in a monomeric state during translation. In comparison, nascent M72 migrated in a high-molecular mass complex with TRiC (Fig. 2.6D). However, native M72 did not accumulate as a free monomer consistent with its migration on a denaturing gel (Fig. 2.6E), but instead showed a diffuse band ranging from approximately 100-150 kDa, suggesting that M72 forms an oligomer.


A
3XFLAG-
Con
M72
250
150
90
75
50
37
25
20
*
*
B
M72-3XFLAG
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HA-Lsm 14a
kDa
1 2 3 4 5 6 7 8
IP:FLAG IB:HA
IP:FLAG IB:FLAG
WCL IB:HA
WCL IB:FLAG
WCL IB:ß-actin
C
Input 0.5%
M72-3XFLAG
FLAG
GAPDH
IP: CCT1+CCT8
(-)
EDTA
ATP+Mg2+
Mg2+
CCT7
FLAG
D
GFP
Human ß-actin
MCMV M72
Time (min): 5 10 20 5 10 20 5 10 20
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1236
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720
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242
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GFP
Human ß-actin
MCMV M72
Time (min): 5 10 20 5 10 20 5 10 20
75
50
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25

Figure 2.6: M72 associates with and is a substrate of the TRiC/CCT complex.

A) Coomassie stained SDS-PAGE gel of FLAG-immunoprecipitates from NIH3T3 fibroblasts transfected with M72-3XFLAG or empty vector control (Con). White arrow indicates M72-3XFLAG. Black arrowhead and boxed region denote region of differentially enriched bands excised and further analyzed by LC-MS/MS (Table 1). Asterisks indicate additional regions of potential differentially enriched proteins. B) IB analysis for HA, FLAG, and actin in IP and WCL from HEK293Ts co-transfected with M72-3XFLAG and HA-epitope tagged TRiC/CCT or control as indicated. C) IB analysis for CCT7 and FLAG from TRiC/CCT substrate assay. HEK293T cells were transfected with M72-3XFLAG, whole cell lysates collected, aliquoted into 4 parts and incubated with EDTA, $MgCl_2$ with or without ATP, or mock treated. Aliquots were subjected to IP with a mixture of TCP1/CCT1 and CCT8 antibodies, separated on an SDS-PAGE gel and immunoblotted with the indicated antibodies. D-E) Native (D) and SDS- (E) PAGE of ^{35}S-methionine (Met)-labelled green fluorescent protein (GFP), β-actin, and MCMV M72 translated for the intervals shown in rabbit reticulocyte lysates (RRLs). Green arrow indicates GFP, the grey arrow indicates actin, the white arrow indicates M72, and the black arrow indicates TRiC/CCT associated proteins.

2.2.8 M72 forms self-associating oligomers in cell culture.

To determine whether M72 forms an oligomer, expression constructs for full-length and C-terminal truncations of M72-3XFLAG (Fig 2.7A) were co-transfected with GFP-M72 into HEK293T cells. Immunoprecipitation followed by immunoblotting revealed that GFP-M72 co-immunoprecipitates with full length M72-3XFLAG, suggesting that M72 forms homo-oligomers both *in vitro* (Fig. 2.6D) and *in vivo* (Fig. 2.7B). Binding of M72-3XFLAG to GFP-M72 is relatively comparable upon deletion of the C-terminus to amino acid 282, but is markedly diminished for M72 $^{(1-253)}$ and M72 $^{(1-226)}$. Although formally possible that the diminished binding upon deletion of aa 226-283 is due to modest expression, it is worth noting that M72$^{(1-226)}$ and M72$^{(1-253)}$ are expressed as well as M72$^{(1-339)}$, M72$^{(1-311)}$, and M72$^{(1-282)}$, yet consistently co-IP less GPF-M72, suggesting that the region of M72 encompassing aa 226-283 are important for efficient oligomerization. M72$^{(1-199)}$ co-precipitates with GFP-M72 as well as full length M72, but this binding is substantially reduced for fragment 1-172 and 1-136, and is completely lost for the smallest fragment, 1-107. Together, these results indicate that regions of M72 from aa 172-199 and aa 226-282 contribute to M72 oligomerization.

To determine whether the ability of M72 mutants to oligomerize correlates with association of M72 with the TRiC/CCT complex, expression constructs for full-length and a subset of C-terminal truncations of M72-3XFLAG (Fig. 2.7A) were co-transfected with HA-CCT8 into HEK293T cells. Immunoprecipitation followed by immunoblotting confirmed that full length M72-3XFLAG could co-IP with CCT8, and M72 $^{(1-199)}$ could co-IP similar amounts of CCT8 as WT (Fig. 2.7C). It also revealed that M72 $^{(1-311)}$,

which showed a higher level of oligomerization (Fig. 2.7B), also precipitated more CCT8 than WT M72. M72 mutants defective for oligomerization were similarly diminished in their capacity to co-IP CCT8. Together, these data indicate that M72 forms homo-oligomers (Fig. 2.7B) which correlates with its ability to associate with components of the TRiC/CCT complex (Fig. 2.7C).

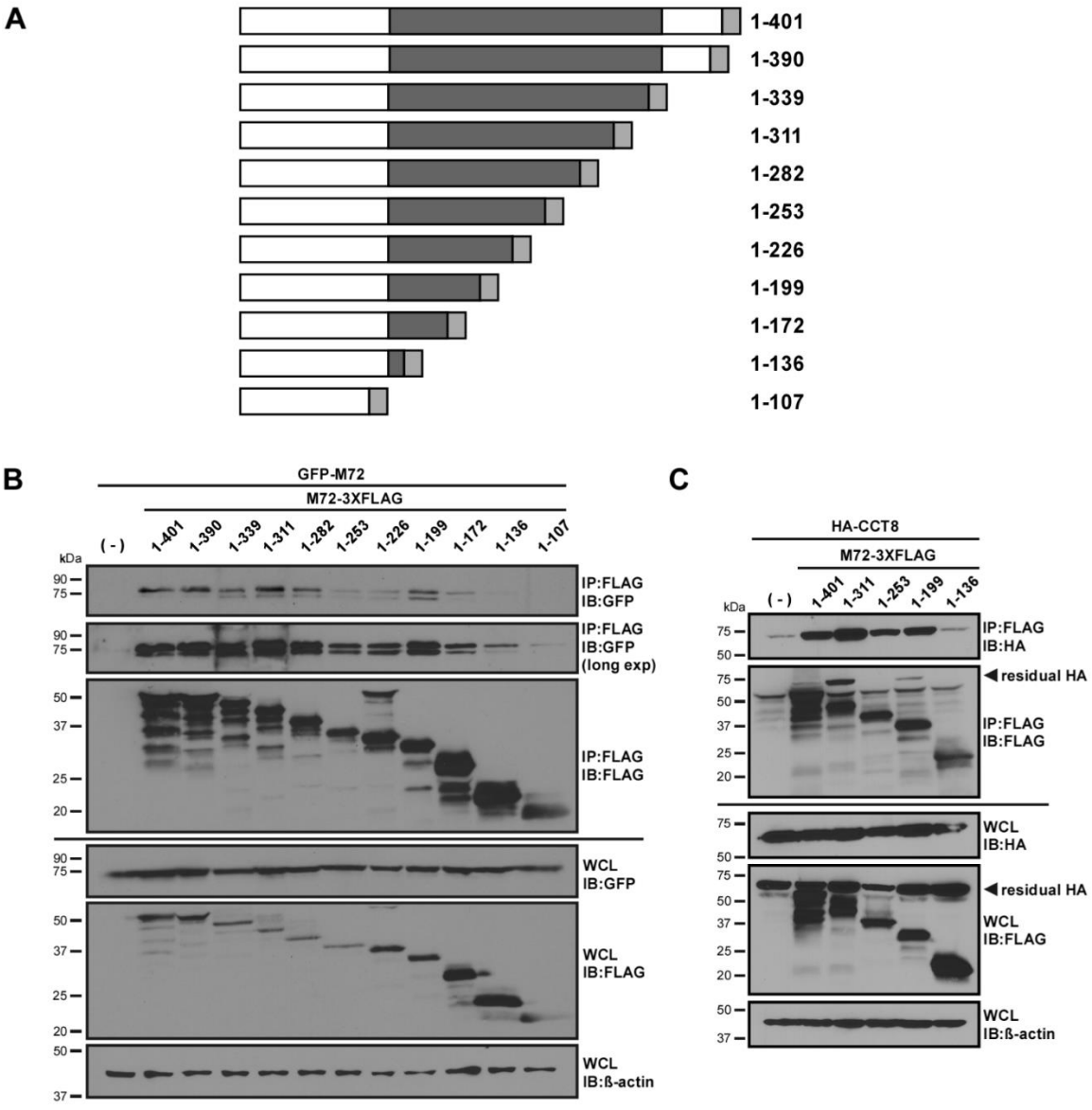

Figure 2.7: M72 forms self-associating oligomers in cell culture.

Schematic diagram of epitope-tagged M72 carboxy-terminal truncation mutants. Dark grey box indicates putative 'dUTPase' domain. Light grey box indicates 3XFlag epitope tag. Numbers denote amino acid numbers. B) IB analysis for FLAG, GFP, and actin in IP and WCL from HEK293T cells co-transfected with eGFP-M72 and the indicated 3XFlag-tagged M72 plasmids. C) IB analysis for FLAG, HA, and actin in IP and WCL from

HEK293T cells co-transfected with HA-CCT8 and the indicated 3XFlag-tagged M72 plasmids.

2.3 DISCUSSION

In this study, I provide an initial characterization of the role of MCMV M72 gene during infection *in vitro* and *in vivo*. Although related to herpesvirus dUTPase enzymes, M72 does not possess intrinsic enzymatic activity (Fig. 2.1), and likely plays no direct role in supporting nucleotide biogenesis during infection. Genetic disruption unveiled a function for M72 in virus replication in fibroblast and endothelial cell lines in culture (Figs. 2.2 and 2.3). In addition, M72 promotes acute virus replication in the early stage of infection in a natural host (Fig. 2.4A). Interestingly, mutation of M72 had little effect on the capacity of MCMV to disseminate to the salivary glands of infected animals (Fig. 2.4C-D). M72 protein is expressed as early as 6 h.p.i. with increasing levels up to 48 hours. Additional, faster migrating M72 isoforms are also detected and accumulate throughout infection (Fig. 2.5C). M72 associates with, and is a substrate of, the eukaryotic TRiC/CCT chaperonin complex (Fig. 2.6). Like other well-characterized TRiC/CCT substrates, such as actin [201], M72 forms oligomers or higher-order aggregates *in vitro* and *in vivo*. This interaction is mediated by a bipartite region of the central portion of M72. Although the specific function of M72 during infection remains unclear, my results indicate complex viral and host regulation of M72 protein expression during infection, and demonstrate that M72 contributes to MCMV replication and pathogenesis.

The dUTPases are classified into three subgroups based on their oligomerization states:, monomeric, dimeric and trimeric. Avian and mammalian herpesvirus dUTPase enzymes are exclusively monomeric, and bioinformatics analysis suggest that trimeric and monomeric dUTPases contain five conserved motifs for catalytic activity [206]. Herpesvirus dUTPase homologs also have an additional motif 6 of unknown function [94, 100, 206]. MCMV M72 was designated as a dUTPase homolog based on limited sequence similarity and positional homology with other herpesvirus dUTPase homologs [13]. Among human beta herpesvirus dUTPase homolog, HCMV UL72 and Human Herpesvirus 6A (HHV-6A) U45 are catalytically inactive [111, 191]. My results confirm that, like other beta herpesvirus dUTPase homologs, M72 is not a functional enzyme (Fig. 2.1). Moreover, an examination of the M72 protein sequence shows a loss of the 5 motifs necessary for enzymatic activity and only has the herpesvirus conserved motif 6 [100]. Although many other herpesvirus dUTPase homologs also fulfill enzyme-independent roles [107-110], my results indicate that M72 plays an exclusively alternate role during infection.

Previous functional profiling studies of the entire HCMV genome indicated that HCMV UL72 is non-essential for replication [207, 208], and a clean UL72 deletion mutant [191] has a modest replication defect in cell culture. My examination of MCMV M72 mutant infection revealed that M72 contributes to virus replication in murine fibroblasts and endothelial cells, similar to the results for HCMV UL72. These results are consistent at both high and low multiplicities of infection (Fig. 2.3 A, B, E, F)),

suggesting that the defect is MOI-independent. However, I also observed that M72 mutant viruses produced yields comparable to or even higher than WT MCMV in murine macrophage cell lines (Fig. 2.3 C and G) and BMDMs (Fig. 2.3 D). This result may be indicative of a tissue-specific contribution of M72 during viral infection.

In the context of a natural host, the MHV-68 dUTPase, ORF54, is required for establishment of latency, but dispensable for acute replication in lungs of infected mice [110]. Additionally, pseudorabies virus lacking its dUTPase homolog, UL50, is also attenuated for virulence and replication in infected swine [15]. However, there are no reports of the contribution of beta herpesvirus dUTPase homologs to infection in the natural host. I show here that M72 augments virus replication during acute replication in spleen at early times post infection (Fig. 2.4). Interestingly, M72Stop mutants effectively disseminated to the salivary glands of infected animals, similar to WT virus (Fig. 2.4C-D). Thus, despite early acute defects in replication seen *in vivo* (Fig. 2.4A), M72-deficient viruses can infect and replicate in the disseminating cell population, showing no defects at later times post infection. Mononuclear phagocytes are the main cell type that contributes to dissemination of MCMV [209], likely after acquiring virus from susceptible cells at sites of primary infection. This result is consistent with my findings in cell culture where there is a modest defect in viral replication in endothelial cells and fibroblasts, but no impairment in the capacity of these viruses to infect and grow in macrophage/monocyte cells (Fig. 2.3). Although not an essential gene, M72 appears to

alleviate barriers to replication in some types of cells. Future studies will provide insights into the host restriction mechanisms overcome by M72.

HCMV UL72 and MCMV M72 have been classified as late gene products [191, 195] based on Northern blot analyses. However, using an epitope-tagged virus, M72 protein expression was observed as early as 6 h.p.i. and this expression is unaffected by addition of PFA (Fig. 2.5C). By comparison, I observed diminished expression of glycoprotein B/M55, a late gene, in the presence of PFA, indicating that drug treatment was effective. The expression pattern of M72 protein more closely mimics that of the m112/3 (E1) protein, a well-known delayed-early gene. Thus, M72 is likely a leaky-late gene product, requiring delayed early protein function, but not requiring viral DNA replication.

Another unanticipated result was the observation of faster migrating FLAG-epitope tagged species by SDS-PAGE analysis from cells infected with MCMV-M72.3XFlag. A similar expression profile, including the faster migrating bands, was observed in infected murine endothelial (SVEC) and macrophage (RAW264.7) cell lines (data not shown), suggesting this phenomenon is independent of cell type. An additional independent HA-epitope tagged M72 recombinant virus displays similar faster migrating bands (data not shown), indicating these bands are not an artifact of the FLAG epitope tag. Initial investigations suggest that that these faster migrating M72 protein isoforms are similar in size to proteins translated from internal methionine positions within the M72 annotated ORF. HCMV UL136 is expressed as multiple protein isoforms and a

detailed characterization of the different isoforms using independent recombinant viruses revealed novel functions for each [210-212]. These isoforms are produced by a complex transcriptional program that uses distinct transcription initiation sites for each isoform [211]. The M72-M75 region of the MCMV genome is transcriptionally complex [193, 195]. However, there do not appear to be distinct transcriptional start sites or alternative splicing for the M72 transcript. We also observe multiple M72 proteins isoforms being expressed following transfection of plasmids encoding the M72 ORF (Fig. 2.1A, 2.6B, 2.7B), indicating that this is not a function of viral infection or replication. M72StopS and M72StopN mutants would be predicted to interrupt some, but not all of the protein isoforms observed by western blot analysis. M72StopS and M72StopN would be predicted to terminate translation between the 3rd and 4th methionine within the ORF. Thus, the full length and two largest possible isoforms would be interrupted by these mutations. However, it is formally possible that additional downstream ORFs remain open and could be expressed during infection. However, I show that interruption of M72 by the –StopS and –StopN mutations are sufficient to impair viral replication *in vitro* and *in vivo*, indicating that longest isoforms of M72 interrupted by both mutations have important functions during infection.

Previous attempts to identify interacting partners of MCMV proteins using a yeast two-hybrid screen showed that M72 engages multiple other viral proteins [213]. Since there are no reports of host factors that interact with M72, I used a proteomics approach to identify cellular proteins that bind M72. Identification of such proteins may

provide clues to M72 function. I identified and confirmed the interaction of M72 *in vitro* and *in vivo* with components of the eukaryotic TRiC/CCT complex (Fig. 5). TRiC/CCT is a well characterized eukaryotic chaperonin and contributes to protein folding of an estimated 5-10% of the cellular proteome [126]. Our original LC-MS/MS analysis identified all eight subunits of the TRiC/CCT complex co-precipitating with M72 expressed in in NIH3T3 cells (Table 1). I repeatedly observed that transfected expression plasmid for M72-3XFLAG was incapable of co-immunoprecipitating CCT5/ε in HEK293T cells. Whether this is an artifact of overexpression or indicative of a cell line difference, is unclear. Multiple examples of viruses that engage the TRiC/CCT complex have been described, including EBV [144], influenza virus [199], rabies virus [139], and reovirus ([200]). My experiments provide evidence that MCMV M72 is likely another viral client of the TRiC/CCT complex (Fig. 2.6).There is no consensus sequence recognition recognized for TRiC/CCT client proteins. However, some biochemical features, such as β-sheets content, aggregation propensity and formation of higher-order complexes are associated with TRiC/CCT binding [126]. Little information is available about the structure of M72. Previous bioinformatics studies [100], as well as initial bioinformatic analysis using structure prediction tools, suggest the presence of multiple β-sheets in M72. Moreover, my data indicates that M72 is capable of forming higher-order oligomers or aggregates (Fig. 2.6D, 2.7B), and this feature correlates with M72's ability to associate with TRiC/CCT subunits (Fig. 2.7C), further supporting M72 as a substrate of the TRiC/CCT complex. Since M72 augments virus replication and is not an

essential MCMV gene, identification of other MCMV (and HCMV) essential gene products as TRiC/CCT clients may provide the rationale for development of TRiC/CCT inhibitors as potential antiviral compounds.

In this report, I characterized the previously unexplored MCMV protein M72. Previous studies have established roles for other designated dUTPase genes among herpesviruses, and future efforts will focus on identifying the specific molecular mechanism of M72 function during infection. It will be important to determine whether the multiple isoforms of M72 protein have distinct functions during infection and how each contributes to pathogenesis. Contribution and characterization of host proteins identified here as candidate M72-interacting proteins, will provide insight into the specific function of M72 during MCMV infection.

Chapter 3: Murine Cytomegalovirus M72 co-opts components of CCR4-NOT transcriptional complex and contributes to viral replication[3]

3.1 INTRODUCTION

Carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) is a large multi-subunit complex that is highly conserved across eukaryotes [145, 146]. It has eight core subunits in mammalian cells and nine in yeast. The core complex in humans consists of CNOT1, CNOT2, CNOT3, CNOT4, CNOT6/6L, and CNOT7/8 and CNOT9 subunits [147, 148]. Additional subunits in the human CCR4-NOT complex include CNOT10, CNOT11 and TAB182, although, association with these depends on conditions [147, 150, 151]. This complex plays an important role in multiple processes. Transcriptional regulation, protein modification and deadenylation of mRNAs are some well-studied functions of this complex.

CNOT1 is the largest subunit of the CCR4-NOT complex and is involved in structural assembly [154]. CNOT1 lacks enzymatic activity however; it is involved in diminishing deadenylase activity in cell. CNOT1 depletion is also implicated in turnover of processing bodies (P bodies), cytoplasmic structures, involved in RNA turnover in the cell [156]. CNOT1 via its interaction with RNA-binding proteins mediates transcription repression [150, 158]. CNOT2 possesses a conserved NOT-box at its carboxyl terminus. Although CNOT2 is not catalytically active by itself, it contributes to deadenylase activity in cells [156]. CNOT6L encodes a conserved deadenylase domain at its carboxyl terminus and a leucine-rich-repeat (LRR) domain at its amino-terminus [163]. The paralog CNOT6

[3] Following authors contributed to experiments in this chapter – Sandhya Gopal (SG), Amanda Y. Xia (AX) and Jason W. Upton (J.W.U.). SG and JWU designed the experiments. AX and SG performed the experiments. SG wrote this dissertation chapter.

is not associated with CNOT6L in the same complex [151], and these proteins are mutually exclusive. Human CNOT6L prefers poly(A) RNA in contrast to poly(A) DNA for activity in *in vitro* deadenylation assays revealing substrate specificity [214]. This proteins has a role in cell proliferation in murine fibroblasts [165]. CNOT6L was identified as a candidate that regulates PTEN, a tumor suppressor protein. In addition, copy number losses in CNOT6L were found in human colon adenocarcinoma samples [215].

The 182-kDa Tankyrase binding protein (TAB182) or Tankyrase 1 binding protein 1 (TNKS1BP1) was previously identified as a component of the CCR4-NOT complex [151] but not in all condition [150]. TAB182 was primarily recognized in a yeast-two hybrid screen as an interacting partner and novel acceptor of Tankyrase 1 [216]. Some reports have suggested a role for TAB182 in the DNA damage response [171, 172], but the mechanism is not clear. TAB182 was also suggested to contribute to actin cytoskeleton rearrangement and cancer cell invasion [174]. Little information is available on role of TAB182 in the context of a component of CCR4-NOT complex.

Human Cytomegalovirus (HCMV), a beta herpesvirus, causes significant morbidity and mortality in immunocompromised patients including hepatitis, retinitis, pneumonitis and even death [77]. Congenital HCMV infection is associated with progressive sensorineural hearing loss, mental retardation and other complications [183]. The cost associated with care of HCMV-infected children is prohibitively high. Hence, development of an HCMV vaccine has been designated as a priority in the United States [184]. Identifying and understanding the role of host factors that contribute to beta herpesvirus infection, is important to tackling issues associated with HCMV. Herpesviruses are highly species-specific, which makes it challenging to study the contribution of individual genes in the context of natural host. To facilitate this process, MCMV is a biologically and

genetically similar virus utilized in mice as a small animal model. Mice are easily available, cost-effective and well-characterized model system to study CMV biology [80]. Additionally, MCMV and HCMV are genetically similar with many homologues genes [187, 188].

My studies have focused on a core MCMV gene M72, previously designated as a deoxyuridine 5' triphosphate pyrophosphatase (dUTPase) homolog [189]. In my previous report, I identified M72 as a catalytically inactive as a dUTPase homolog, opening up the possibility for alternate roles in MCMV replication. I constructed two-independent stop mutants of M72, (M72StopS and M72StopN) and showed that M72 augments the growth of virus in culture, in a cell-type dependent manner. I demonstrated that M72 augments virus replication in the natural host in the early acute phase of infection and has a complex protein expression pattern during infection. To begin to understand the function of M72, I took a proteomics approach. I identified subunits of a eukaryotic chaperonin complex TRiC/CCT as M72 candidate interacting proteins and established that TRiC/CCT contributes to M72 protein folding [1].

Here I have continued investigating other M72 interacting host proteins identified from the same proteomic screen. I had previously reported enrichment of CNOT2 and CNOT6L in M72-3XF samples [1] and investigated whether other components of the hetero-oligomer CCR4-NOT transcription complex were present. I analyzed data from additional enriched bands and identified CNOT1 and TAB182 as novel candidate-interacting proteins. Co-immunoprecipitation experiments helped validate and map the interaction of M72 with components of the CCR4-NOT transcription complex.

To elucidate the role of this complex during viral replication, I silenced CNOT1 and TAB182 and evaluated MCMV growth. I found that CNOT1 is necessary for wild type

(WT) MCMV infection at both high and low multiplicities of infection (MOI). The M72StopS mutant grew to levels comparable to WT virus later during high (MOI) infection but was attenuated compared to WT in low MOI infections. TAB182 silencing had no effect on WT and M72StopS replication compared to the relevant control. I found that CNOT1 and TAB182 protein expression was comparable between WT and M72StopS mutant during infection. This is the first report to demonstrate the role of CNOT1 relevant to MCMV biology. Thus, M72 is involved at least partially in CNOT1 mediated function during MCMV infection.

3.2 RESULTS

3.2.1 Identification of components of CCR4-NOT transcriptional complex as interacting partners of MCMV M72

To understand the function of M72, I utilized the data from the proteomics approach previously described [1]. Briefly, M72-3XFlag or control plasmid was transfected into NIH3T3 fibroblasts. FLAG-immunoprecipitates were prepared, resolved by 10% SDS-PAGE and stained. Multiple bands were observed in the M72-3XFLAG sample relative to the vector control (Fig. 3.1). A region of each lane in which multiple bands were differentially enriched, corresponding to approximately 55-75 kDa, and 150-250 kDa were excised from each sample, subjected to in-gel tryptic digestion and LC-MS/MS analysis. Comparison with the UniProt mouse reference genome helped to generate a list of 109 total unique proteins. I excluded proteins of low confidence representation, low abundance, those enriched in the control sample and subunits of TRiC/CCT complex previously described [1]. A significant enrichment for components of a large multi-subunit complex called the CCR4-NOT transcriptional complex and a protein called Tankyrase-1 Binding Protein (TAB182) was observed in M72-containing immunoprecipitates. I had previously

reported two subunits of the CCR4-NOT transcription complex, specifically CNOT2 and CNOT6L were enriched in the 55-75 kDa bands. These and other proteins identified in bands from the 150-250 kDa region were used to generate a more comprehensive list of candidate proteins (summarized in Table 3.1).

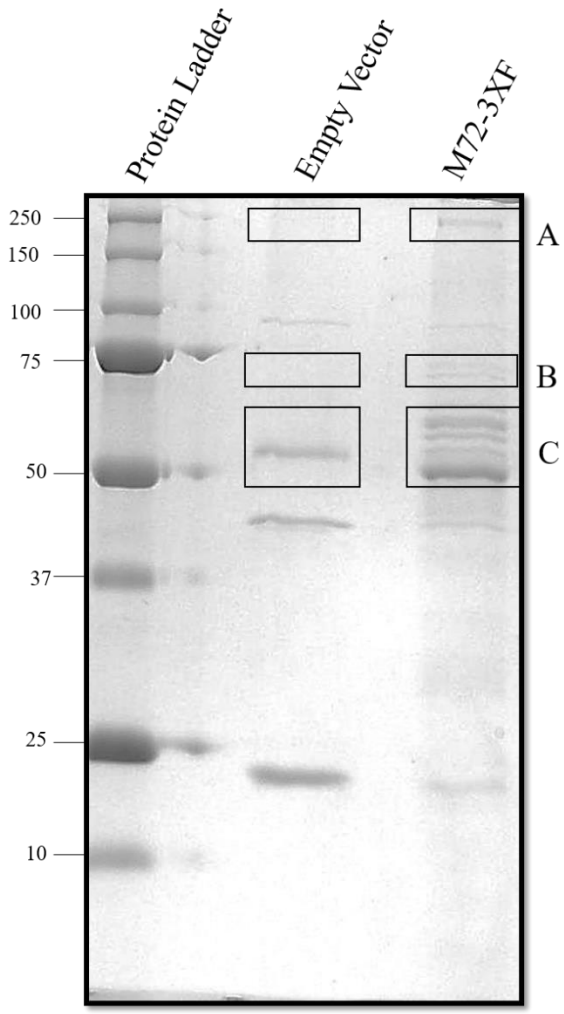

Figure 3.1: Analysis of immunoprecipitates from M72 transfected cells.

NIH3T3 fibroblasts were transfected with M72-3XFLAG and the corresponding empty vector control. Whole cell lysates were immune precipitated with the FLAG antibody, eluted, fractionated on a 10% SDS-PAGE gel and stained with Coomassie Blue stain. The differential bands on the gel in the M72-3XF lane and the corresponding area of Empty Vector control lane were subjected to LC-MS/MS analysis. The letters A, B, C indicate gel fractions for M72-3XFLAG and corresponding Empty Vector control.

Identified protein	UNIPROT Accession	kDa	Gel fraction	Peptide spectral counts (PSM)	
				Control (EV)	M72-3XF
CCR4-NOT transcription complex subunit 1	CNOT1_MOUSE	240	A	1	352
182 kDa Tankyrase-1 binding protein	TB182_MOUSE	182	A	0	246
CCR4-NOT transcription complex subunit 1	CNOT1_MOUSE	?	B	1	121
182 kDa Tankyrase-1 binding protein	TB182_MOUSE	?	B	0	121
CCR4-NOT transcription complex subunit 2	CNOT2_MOUSE	60	C	0	34
CCR4-NOT transcription complex subunit 6-like	CNO6L_MOUSE	63	C	0	18

Table 3.1: Expanded candidate M72-interacting proteins identified by mass spectrometry.

List of candidate proteins generated by subjecting the gel fragments indicated in Figure 3.1 to LC-MS/MS analysis. Candidate proteins, their molecular mass, gel fragments from which they were obtained, and total peptide spectral counts are indicated in the table.

3.2.2 Validation and mapping of interaction of MCMV M72 with components of CCR4-NOT transcriptional complex

To confirm the results of the MS experiment, I performed co-tranfections, co-immunoprecipation and immunoblotting experiments in the HEK293T cell line. I utilized full-length Human (Hs) GFP-CNOT1, GFP-CNOT2 and GFP-CNOT6L expression vector constructs along with plasmids expressing M72-3XFLAG full-length and a series of carboxyl-terminal truncations of M72 (described earlier [1]). My MS experiment was performed in NIH3T3 murine fibroblast cells, with the human expression constructs for transient transfection assays. The human GFP-CNOT subunit expression constructs were readily available and the murine and human CNOT 1, 2 and 6L subunit proteins were almost identical based on pairwise sequence alignment (Table 3.2) so these plasmids were used for transient transfection assays.

I co-transfected GFP-CNOT1 and M72-3XFLAG full-length along with a series of carboxyl terminal truncations of M72 (Fig. 3.2 D) and performed co-immunoprecipitation. After immunoblotting, I observed that GFP-CNOT1 co-immunoprecipitated with full-length M72-3XFLAG, but not with the corresponding FLAG empty vector control construct (Fig. 3.2A). This suggests that GFP-CNOT1 interacts with M72-FLAG specifically. The binding of $M72^{(1-390)}$ was comparable to full-length $M72^{(1-401)}$. However, there was a decrease in co-immunoprecipitation of GFP-CNOT1 with $M72^{(1-339)}$, even when the expression of GFP-CNOT1 in the whole cell lysate was comparable in both samples. Therefore, the M72 region between amino acids (aa) 340 to 390 augments interaction with CNOT1. Binding of M72-3XFLAG to GFP-CNOT1 was comparable for carboxyl terminal aa deletions from $M72^{(1-339)}$ to $M72^{(1-199)}$. A markedly diminished co-immunoprecipitation of GFP-CNOT1 with $M72^{(1-172)}$ and completely loss for fragments (1-136) and (1-107) was observed. The expression of GFP-CNOT1 was comparable in

corresponding whole cell lysates transfected with M72-3XFLAG fragments (1-199), (1-172), (1-136) and (1-107). These results suggest that M72 aa 173 to 199 additionally augment interaction with GFP-CNOT1, and, that M72 aa region 137 to 172 is necessary for interaction with GFP-CNOT1.

I performed a similar co-transfection and co-immunoprecipitation experiments for Human GFP-CNOT2 with M72 full length and carboxyl-terminal truncations. Immunoblotting revealed that GFP-CNOT2 co-immunoprecipitated with full-length M72-3XFLAG (Fig. 3.2B), suggesting interaction, and this was comparable to the $M72^{(1-172)}$ construct with a deletion of aa 173 to 401 at the carboxyl terminus. GFP-CNOT2 co-immunoprecipitation was markedly decreased for both $M72^{(1-136)}$ and $M72^{(1-107)}$ even when its expression in the two corresponding whole cell lysate sample was comparable with $M72^{(1-172)}$, $M72^{(1-199)}$ and $M72^{(1-226)}$. Thus the M72 region between aa 137 to 172 is likely necessary for interaction with GFP-CNOT2.

In the context of human GFP-CNOT6L, I performed a comparable experiment as described above with M72 full-length and carboxyl-terminal truncations. We observed that GFP-CNOT6L co-immunoprecipitated with M72-3XFLAG full-length (Fig. 3.2C) suggesting binding. This interaction was comparable with the $M72^{(1-172)}$ construct, but completely lost for fragments $M72^{(1-136)}$ and $M72^{(1-107)}$. Therefore, aa 137 to 172 are needed for efficient binding of M72-3XFLAG with GFP-CNOT6L. An improved co-immunoprecipitation of GFP-CNOT6L with $M72^{(1-390)}$ compared to M72 full-length was not reproducible (data not shown). These results (summarized in Fig. 3.2 E) suggest the interaction of M72 with components of CCR4-NOT transcription complex is complex and involves multiple parts of the protein.

Protein pairs compared	Pairwise % identity[a]
Hs and Mm CNOT1	99.3
Hs and Mm CNOT2 (full)[b]	96.6
Hs and Mm CNOT6l	98.0

Table 3.2: Pairwise percentage identity at amino acid level calculated with Geneious 6.1.8.

[a]Identity is the percentage of amino acids with a direct match in the alignment.
[b]CNOT2 protein has multiple isoforms, so utilized full-length protein sequence

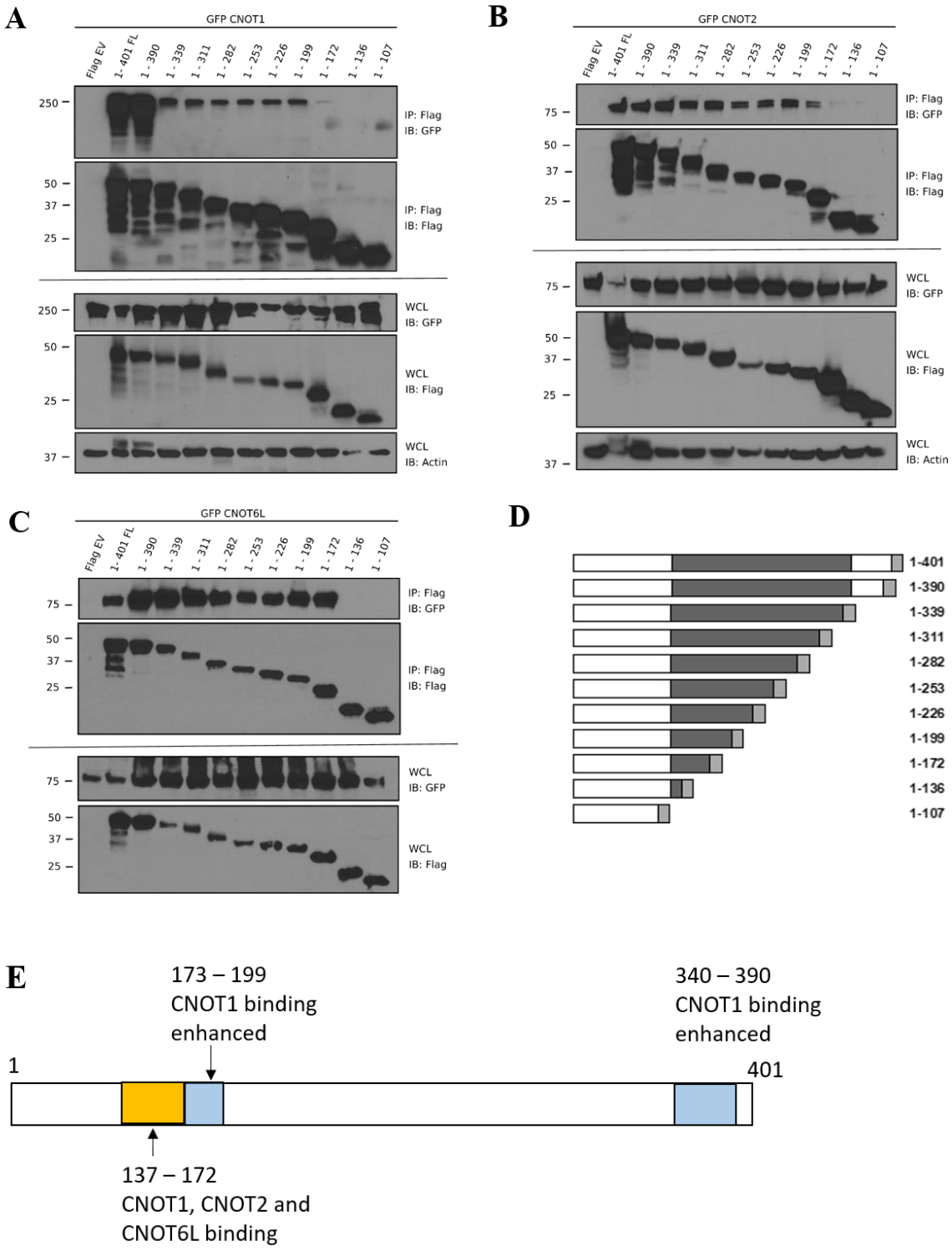
A
GFP CNOT1
Flag EV
1-401 FL
1-390
1-339
1-311
1-282
1-253
1-226
1-199
1-172
1-136
1-107
250
50
37
25
IP: Flag
IB: GFP
IP: Flag
IB: Flag
WCL
IB: GFP
WCL
IB: Flag
WCL
IB: Actin
B
GFP CNOT2
75
C
GFP CNOT6L
D
1-401
1-390
1-339
1-311
1-282
1-253
1-226
1-199
1-172
1-136
1-107
E
173 – 199
CNOT1 binding
enhanced
340 – 390
CNOT1 binding
enhanced
1
401
137 – 172
CNOT1, CNOT2 and
CNOT6L binding

Figure 3.2: Validation and mapping of MCMV M72 interaction with components of the CCR4-NOT transcription complex.

A, B and C) IB analysis for FLAG, GFP, and actin in IP and WCL from HEK293T cells co-transfected with the indicated 3XFlag-tagged M72 plasmids and GFP-CNOT1 (A), GFP-CNOT2 (B) and GFP-CNOT6L (C). D) Schematic diagram of epitope-tagged M72 carboxy-terminal truncation mutants. The dark grey box indicates putative 'dUTPase' domain. The light grey box indicates the 3XFlag epitope tag. Numbers denote amino acids. E) Schematic diagram summarizing the interaction of M72 with components of CCR4-NOT complex based on panels A), B) and C).

3.2.3 Knockdown of CNOT1 reduces WT to comparable level as M72StopS mutant at late time during replication.

I identified and validated components of the CCR4-NOT complex as interacting proteins of MCMV M72. Therefore, I sought to understand their role in viral replication by transiently disrupting the expression of CNOT1 and TAB182 independently in murine fibroblasts and investigating their effect on viral yield. I previously generated and characterized two independent recombinant viruses designated as M72StopS and M72StopN mutants. These mutants have engineered stop codons inserted within the M72 gene. I identified that both are attenuated comparably by about tenfold at both high and low multiplicity of infection in murine fibroblasts (NIH3T3) compared to WT MCMV [1]. Infection with WT virus would allow me to assess the contribution of CNOT1 and TAB182 to MCMV infection. Additionally, infection with any one of the M72 mutants will facilitate understanding of the role of M72 in virus biology. Therefore, I used WT and M72StopS recombinant virus for infections.

In a CNOT1-knockdown background, I infected at both high (Fig. 3.3 A) and low (Fig. 3.3C) multiplicities of infection (MOI) and collected samples at the indicated time points. Quantitation of viral yields showed that at the high MOI of 5 PFU/cell WT MCMV was attenuated by about 100-fold at 24 hours post infection (h.p.i.) and by about 10 fold at 48 h.p.i. compared to the scrambled siRNA control suggesting that CNOT1 contributes to MCMV infection (Fig. 3.3A). Using a low MOI (of 0.05 PFU/cell), revealed similar trends where the WT virus grew at lower levels in CNOT1 knockdown cells compared to the scrambled siRNA transfected cells (Fig. 3.3C). Taken together, these results indicate that CNOT1 contributes to WT MCMV replication independent of replication kinetics.

To understand the role of M72 in the context of CNOT1, I assessed growth of M72StopS recombinant virus. At the high MOI infection in CNOT1 knockdown cells, the

mutant was attenuated compared to WT by about five fold at 24 h.p.i. However, at 48 h.p.i., the replication of the M72StopS mutant was comparable to wild type virus in CNOT1 knockdown background (Fig. 3.3A). Importantly, in the scrambled siRNA control cells, the M72StopS is defective compared to WT virus at both 24 and 48 h.p.i. This suggests that the modest difference in viral titers between WT and M72StopS is alleviated upon knockdown of CNOT1 later during replication. The low MOI experiment (Fig. 3.3C) yielded slightly different observations. M72StopS replication in this setting was attenuated compared to WT MCMV both in the control cells in the CNOT1 knockdown background at 24, 48 and 72 h.p.i. (Fig. 3.3C). However, it is noted that the attenuation of M72StopS compared to WT in control cells is about 10-fold, and this difference is reduced to about 3-fold in CNOT1 knockdown background in a low MOI setting. Knockdown of CNOT1 was confirmed for experiments at both MOIs (Fig. 3.3B and D).Taken together, these results suggest that the knockdown of CNOT1 reduces WT replication to same level as M72StopS in a replication kinetics dependent manner. Additionally, CNOT1 mediated biology is involved partially in M72 function.

Similarly, NIH3T3 cells were transiently transfected with TAB182 (Fig. 3.3 E) and in parallel control (SCR) siRNAs, infected with WT and M72StopS MCMV at high MOI of five PFU per cell and viral yields determined. WT virus grew at about the same level in control and TAB182 knockdown cells (Fig. 3.3E), suggesting that TAB182 does not contribute to MCMV replication. M72StopS replication was attenuated compared to WT virus by about 10-fold in both control and TAB182 knockdown cells (Fig. 3.3E). Western blotting confirmed TAB182 knockdown (Fig. 3.3F). This indicated that the attenuation of M72StopS in murine fibroblasts is independent of TAB182. A small two-fold attenuation in both WT and M72StopS viruses in TAB182 knockdown background was observed

compared to the scrambled control cells only at 48 h.p.i. However, its role in virus biology remains unclear. Taken together, these results indicate that TAB182 is not involved in both WT and M72StopS MCMV replication in murine fibroblasts.

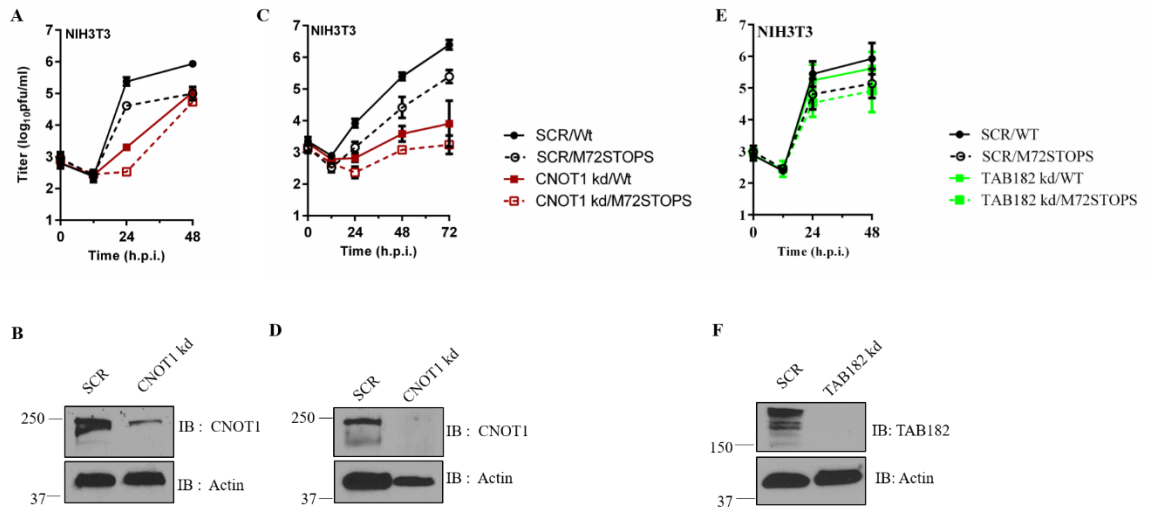

Figure 3.3: Knockdown of CNOT1 reduces WT to comparable level as M72StopS at late time during replication in cell culture.

A and C) NIH3T3 cells were transiently transfected with scrambled (SCR) control or CNOT1 siRNAs E) NIH3T3 cells were transiently transfected with SCR or TAB182 siRNAs. A, C and E) NIH3T3 cells were infected with WT or M72StopS MCMV recombinant viruses at 5 PFU/cell (A and E) or 0.05 PFU/cell (C). Samples were collected at indicated time points and viral yields were determined by plaque assay. B, D and E) NIH3T3 cell lysates were collected in parallel for A, C and E, respectively, at 48 h.p.i. by addition of SDS lysis-buffer, boiled, analyzed on 4-20% TGX SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies. Each data point represents n = 3 replicates.

3.2.4 Expression of components of CCR4-NOT complex is comparable between WT and M72StopS replication.

My earlier results (Fig. 3.3) suggested that CNOT1 subunit is required for WT MCMV infection and that CNOT1 mediated biology is involved partially in M72 function. Therefore, I sought to understand the expression of components of CCR4-NOT complex during virus replication. Murine fibroblasts were either mock infected or infected at high MOI with WT and M72StopS, and cell lysates were collected. Immunoblotting revealed that levels of CNOT1, CNOT2, CNOT7 and TAB182 were comparable between mock, WT and M72StopS at the indicated time points during infection. A decrease in CNOT1, CNOT2 and TAB182 in WT- and M72StopS-infected cells was observed at 48 h.p.i. compared to mock infected cells, but a corresponding actin decrease also was noted. This suggested that it is possibly due to cytopathic effects of viral replication. A modest increase in expression of TAB182 at 24 and 48 h.p.i. was observed in mock, WT- and M72StopS-infected cells compared to zero and 12 h.p.i. but it was independent of M72. Therefore, infection by MCMV does not appear to affect CNOT1, CNOT2, CNOT7 and TAB182 levels upon virus replication or due to M72.

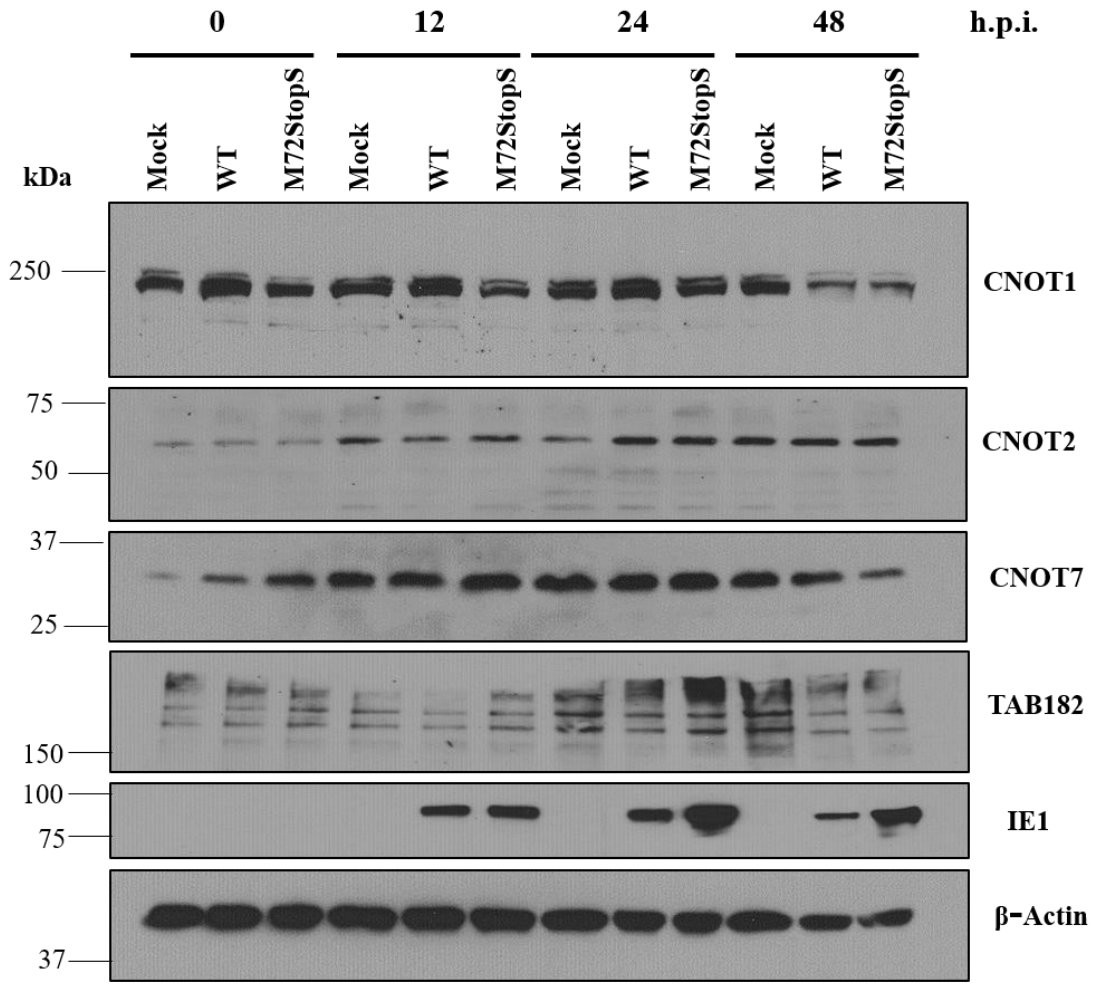

Figure 3.4: CNOT1, CNOT2, CNOT7 and TAB182 are expressed at comparable levels during WT and M72StopS replication in murine fibroblasts.

NIH3T3 cells were infected with mock or WT or M72StopS recombinant viruses at 5 PFU/cell, lysates were collected at indicated time points and analyzed by immunoblotting with indicated antibodies.

3.2.5 M72 associates with CNOT1 during virus replication.

My initial data suggests that M72 and CNOT1 interact in isolation. I next sought to determine if CNOT1 interacts with M72 during virus replication. To answer this I utilized a previously described M72-3XFLAG epitope tagged recombinant virus [1]. Murine fibroblasts were infected with WT and M72-3XFLAG MCMV in parallel at an MOI of 5 PFU/cell for respective virus and lysates were collected at 24 h.p.i. Upon performing FLAG immunoprecipitation (Fig. 3.5A), there is some background binding of CNOT1 to FLAG beads in WT virus infected samples. However, there is a definite enrichment of CNOT1 in the M72-3XFLAG virus infected lysates (Fig. 3.5A). A reverse CNOT1 immunoprecipitation (Fig. 3.5B) demonstrated the presence of M72-3XFLAG specifically in the corresponding virus infected sample and not in the WT MCMV infected sample. This suggests that M72 and CNOT1 interact directly with each other during virus replication.

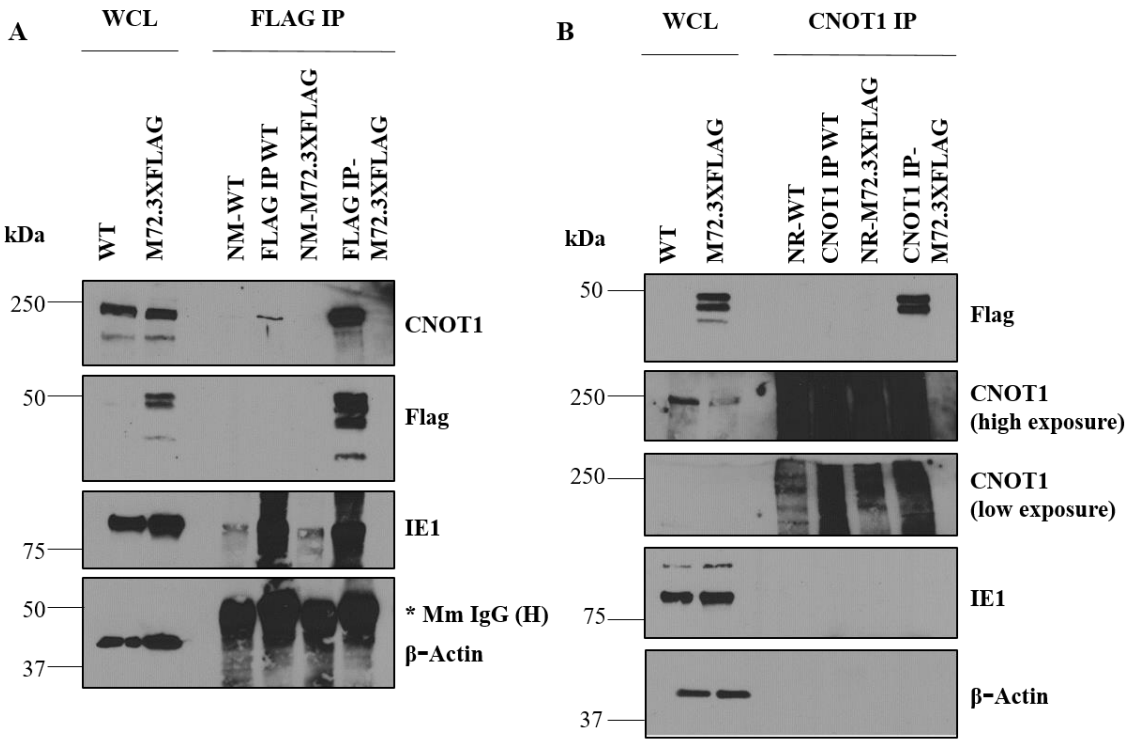

Figure 3.5: M72 associates with CNOT1 during virus replication.

NIH3T3 cells were infected with WT or M72-3XFLAG MCMV in parallel at MOI of 5 PFU/cell and lysates were collected at 24 h.p.i. FLAG A) or CNOT1 B) immunoprecipitation was performed with corresponding normal mouse (NM) A) or normal rabbit (NR) B) IgG controls. Samples were boiled in SDS-lysis buffer, separated on 4-20% TGX SDS-PAGE gels, transferred to nitrocellulose membrane and immunoblotted with indicated antibodies.

3.3 DISCUSSION

In this chapter, I utilized candidate proteins CNOT2 and CNOT6L identified earlier [1] and analyzed additional bands enriched in M72-3XF immunoprecipitates (Fig. 3.1). Components of the CCR4-NOT transcription complex and TAB182 were identified as candidate interacting proteins of MCMV M72 (Table 3.1). I used M72 full length and carboxyl-terminal truncation constructs (Fig. 3.2D) along with Hs CNOT1, Hs CNOT2 and Hs CNOT6L. Co-immunoprecipitation experiments were used to validate the interactions. Association between M72 and components of CCR4-NOT complex were confirmed. I was unable to co-immunoprecipitate other subunits of this complex in the initial mass spectrometry experiment, but it is likely that some other subunits are part of this complex via an indirect interaction. This is the first account of interaction between CCR4-NOT complex components and herpesvirus proteins. In addition, there are very few published reports regarding involvement of this complex with viruses. There are examples of HPV E6 protein interacting with CNOT1, CNOT2 and CNOT3 [179], and adenovirus E1B55K protein co-immunoprecipitating with CNOT1 [178]. Therefore, a precedence for DNA virus interaction with CCR4-NOT complex exists.

I mapped the interaction of M72 with CNOT1 (Fig. 3.2A), CNOT2 (Fig. 3.2B) and CNOT6L (Fig. 3.2C) subunits. Amino acids 173-199 and 340-390 of M72 enhance binding with CNOT1, and amino acids 137-172 of M72 are necessary for binding with CNOT1 (Fig. 3.2A and E). Amino acids 137-172 of M72 are necessary for interaction with CNOT2 (Fig. 3.2B and E) and CNOT6L (Fig. 3.2C and E). These results imply a multi-partite interaction between M72 and CNOT1, whereas a single region of interaction between M72 and CNOT2 and CNOT6L was observed. Taken together, multiple regions of M72 are involved in mediating binding with the entire CCR4-NOT complex. A similar example for

this is seen in the host protein Tob along with BTG. The Tob/BTG families of proteins are transcriptional cofactors and implicated in cell growth and differentiation. Tob is suggested to form a bridge between CNOT1 and CNOT7 whereas BTG has a single region contributing to interaction with CNOT7 [217].

To begin to understand the contribution of components of CCR4-NOT complex on MCMV replication, I transiently silenced CNOT1 and TAB182 in murine fibroblasts, infected these cells with WT and M72StopS recombinant viruses, and evaluated viral growth. Knockdown of CNOT1 diminished WT MCMV replication independent of kinetics of infection (Fig. 3.3A and C). This implies that CNOT1 is a host factor required for MCMV replication. Two illustrations among RNA viruses, HCV [180] and Influenza A virus [181], show that knockdown of CCR4-NOT complex components diminishes viral replication. CNOT1, CNOT2, CNOT3 and CNOT6L are required for HCV replication [180]. In IAV, silencing of CNOT4 subunit diminishes viral replication. CNOT4 subunit is a RING-domain containing E3 ligase and post-translationally modifies the nucleoprotein of IAV, potentially providing structural stability, promoting RNA transcription and replication [181]. The above three examples (MCMV, IAV and HCV) build a case for categorizing CNOT1 as a required host factor. However, in complete contrast, knockdown of CNOT1 promotes adenovirus replication. Though the mechanism is not clear, CNOT1 may promote adenovirus early protein expression [178]. Thus, CNOT1 is a very dynamic host factor in virus replication. Owing to its association with multiple subunits and implications in diverse cellular processes [154], its contribution most likely depends on the type of sub-complex formed with each type of virus. This also implies that CNOT1 is a likely starting point, and continued investigations of other subunits in this complex will delineate their role in MCMV replication.

In the context of M72, we observed that CNOT1 knockdown reduces the replication of WT virus to comparable levels as M72StopS late in viral replication when infected at a high MOI (Fig. 3.3A). Intriguingly, in a low MOI infection, CNOT1 knockdown does bring down WT viral titers to M72StopS (Fig. 3.3C) at least up to the time points tested. The difference in viral replication levels between WT and M72StopS was diminished (Fig. 3.3 C) in the CNOT1-knockdown background. This exciting result indicates a genetic connection between CNOT1 and M72 biology. However, it does not completely explain the attenuated phenotype of the M72StopS recombinant virus. It is possible that other subunits like CNOT2 and CNOT6L, which have not been entirely investigated, have a role to play in M72 biology. Alternatively, an entirely independent host factor might be contributing to M72 biology. A systematic screen to identify cellular determinants interacting with M72 during infection might be one way to approach this issue in the future.

I observed that TAB182 is dispensable for WT MCMV replication and any potential M72 function in murine fibroblasts (Fig. 3.3E). This negative data is important because it suggests specificity to CNOT1-mediated reduction of WT to M72StopS level (Fig. 3.3 A). TAB182 and CNOT1 independent knockdowns have distinct effects on WT and M72StopS replication. Some reports suggest that TAB182 commonly co-immunoprecipitates with components of the CCR4-NOT complex, while others report a conditional effect [150, 151]. Other than its initial identification in the proteomic screen, the co-immunoprecipitation of TAB182 with M72 has not been performed. Additionally, experiments to determine if TAB182 co-immunoprecipitates as a part of the CCR4-NOT complex has not been confirmed. At least in murine fibroblasts, TAB182 does not appear to contribute to MCMV replication. However, since MCMV and HCMV exhibit a wide range of tissue tropism [218], TAB182 may participate in a different cell type. One report

showed that adenovirus E4orf6 protein degraded TAB182 during infection. In addition, knockdown of TAB182 promoted Adenovirus infection [178]. Thus, there is precedence in literature for this novel host factor's involvement in DNA virus replication.

Since MCMV requires CNOT1 during infection, I hypothesized that infection upregulates the expression of components of CCR4-NOT complex. Analysis of protein amounts revealed that CNOT1, CNOT2, CNOT7 and TAB182 levels during high MOI infection are comparable between mock, WT- and M72StopS- infected samples (Fig. 3.4). Levels of other components of the complex, especially CNOT6L, identified in my screen will be examined during MCMV infection.

Utilizing an epitope-tagged M72-3XFLAG recombinant virus, I demonstrated that M72 and CNOT1 interact during MCMV replication. It remains to be investigated if other components of the CCR4-NOT complex, especially CNOT2 and CNOT6L co-immunoprecipitate with M72.

Based on my current results, I believe that M72 co-opts CNOT1 and possibly other components of the CCR4-NOT transcription complex during virus replication. Future work will focus on contribution of CNOT2 and CNOT6L to MCMV infection and M72 biology. The CCR4-NOT, complex has been implicated in multiple cellular processes [154]. CNOT1 and CNOT2 lack enzymatic activity by themselves [147]. However, I identified and validated interaction of M72 with CNOT6L, a subunit with deadenylase activity [147]. I suggest that M72 utilizes its association with CCR4-NOT complex to regulate the turnover of mRNA substrates via poly (A) tail deadenylation and subsequent degradation. Future work will investigate this hypothesis and focus on the mechanism of CNOT1 requirement to MCMV and M72 biology.

Importantly, my results suggest that it is imperative to assess if interaction with components of the CCR4-NOT complex is conserved for HCMV and possibly other members of the herpesviruses. Future mechanistic studies will help address this issue and potentially expand the pipeline of therapeutic approaches to tackle CMV issues.

Chapter 4: Conclusions and Future Directions

4.1 Characterization of MCMV M72 And Identification of TRiC/CCT as a novel host factor

4.1.1 Summary

The goal of my dissertation research was to understand the beta herpesvirus dUTPase homolog, M72, based on the interesting roles among other members of herpesviruses. I showed that M72 is not enzymatically active as a dUTPase implying alternate function/s in replication. M72 is expressed as a leaky-late gene and has a complex expression profile with multiple isoforms. I established for the first time that a beta herpesvirus dUTPase homolog augments virus replication in cell culture and in the natural host. M72 was identified as a client of the TRiC/CCT chaperonin complex and forms homo-oligomers [1].

4.1.2 Future Directions

My result that M72 is not active as a dUTPase was not surprising, since M72 lacks five out of the six conserved catalytic motifs. However, the presence of multiple isoforms during infection was intriguing. My initial examination suggests that isoforms are made from internal methionine positions. This needs to be confirmed in the future by mutagenesis. Generating a panel of internal methionine point mutants and an M72 construct in which all the internal methionine positions are mutated leaving behind only the starting methionine will be valuable reagents. It will be important to build and characterize the phenotype of such an 'internal methionine less' M72 recombinant virus and compare it with the existing M72Stop mutants. Based on bioinformatics predictions, M72 has a nuclear localization signal (NRRKKLKRK) between amino acids 5 and 13 from

the amino-terminus of the protein (utilizing NucPred online tool [219]). This suggests that the full-length protein has a nuclear localization. The question then arises regarding localization of the internal methionine isoforms of M72. A panel of M72 constructs initiating from internal methionine position has been constructed. These existing constructs and the new panel of point mutants could be used to assess the localization of multiple M72 protein isoforms. Further, recombinant viruses corresponding to the internal methionine constructs can be generated and the phenotype of these can be characterized in different cell types.

My data suggests that M72 augments the replication of virus in a cell culture system. The current M72Stop mutants block only the three longest isoforms. This might explain the modest attenuation I observed compared to WT replication. It will be valuable to generate a more refined stop mutant further downstream of M72 than existing ones in which all three reading frames are interrupted, characterize the growth of this new recombinant virus and compare the phenotype with previous M72Stop mutants.

I identified an interaction of M72 with the TriC/CCT complex and showed that the chaperonin complex aids in protein folding. It is possible that other MCMV proteins utilize the chaperonin complex for folding and assembly. Co-immunoprecipitation of the chaperonin complex during infection and subsequent proteomic analysis is one approach to identify viral proteins associating with TRiC/CCT. Extension to HCMV also will be valuable. The TRiC/CCT complex has been studied in the context of protein aggregation disorders [220] and cancer progression [221] and efforts are being made to identify inhibitors of this complex. If essential viral genes are identified in MCMV or HCMV as TRiC/CCT client proteins, this chaperonin complex will likely be added to the list of therapeutic targets for HCMV.

4.2 COMPONENTS OF THE CCR4-NOT COMPLEX AS NOVEL HOST FACTORS CONTRIBUTING TO MCMV REPLICATION.

4.2.1 Summary

Along with other M72 candidate interactions from our previous proteomic screen [1], we identified components of the CCR4-NOT complex. I validated and mapped the interaction of M72 with CNOT1, CNOT2 and CNOT6L. CNOT1 is required for WT MCMV replication and at least partially contributes to M72 biology. **My current working model is that M72 co-opts components of CCR4-NOT complex to mediate its own function (Fig. 4.1).**

4.2.1 Future Directions

I utilized a series of carboxyl-terminal truncations of M72 and mapped the interaction with CNOT1, CNOT2 and CNOT6L. In the future, a complementary set of amino-terminal truncations and/or specific deletions of the indicated M72 interacting regions may be needed. These can be used to confirm my initial mapping experiments. It might also be significant to map the interaction on each of the CCR4-NOT complex subunits with M72 by generating truncations of CNOT1, CNOT2 and CNOT6L and performing reverse co-immunoprecipitations. I have not investigated the role of CNOT2 and CNOT6L in MCMV replication. Transient or stable independent knockdowns of these two subunits and similar experiments (*e.g.*, Fig. 3.3) to evaluate viral growth in WT and M72StopS might be useful. Since my data suggests that expression of the CCR4-NOT components is comparable before or during infection, it will be interesting to assess localization of CNOT1, CNOT2 and CNOT6L subunits and M72 during infection. I speculate that M72 might be co-localizing with at least CNOT1 and some other components of this complex. Localization of the CCR4-NOT complex components upon

infection or during specific time points during viral replication can provide further mechanistic insight. I have described generating the M72-3XFLAG tagged recombinant virus previously [1]. M72-HA and M72-mCherry tagged two independent recombinant viruses also have been generated. Any of these three recombinant viruses can be utilized to assess localization of M72 and the CCR4-NOT complex components. In addition, co-immunoprecipitation experiments during infection in future might yield information on the nature of the CCR4-NOT complex subunits interacting with M72 during virus replication.

CNOT1 and CNOT2 by themselves do not have any enzymatic role. However, these proteins contribute to deadenylase activity in human cells [156]. I have validated and mapped the interaction of M72 with CNOT6L, a subunit with known deadenylase activity [154]. Therefore, **I hypothesize that M72 utilizes components of the CCR4-NOT complex and deadenylates host and/or viral mRNAs during infection (Fig. 4.1)**. There are examples in literature of CNOT1 interacting with specific RNA-binding proteins and mediating turnover of mRNA substrates [150, 158]. It is also likely that M72 interacts independently with an RNA-binding protein. One of the proteins enriched in M72-FLAG precipitates was the RNA-binding motif protein 14 (RBM14). RBM14 contains two RNA recognition motifs, localizes in paraspeckles in the nucleus and functions as a general nuclear coactivator [222]. Since, RBM14 was at low abundance (zero in the empty vector versus 12 in M72-3XFLAG total spectral counts), I did not pursue it here. However, with my current data, it is one of the possibilities to explore. Another speculation is that M72 by itself can bind to mRNAs. If the above-mentioned hypothesis is true, current carboxyl-terminal M72 truncations can be used to map the deadenylase activity and assess if knockdown of CNOT1, CNOT2 or CNOT6L diminishes the activity.

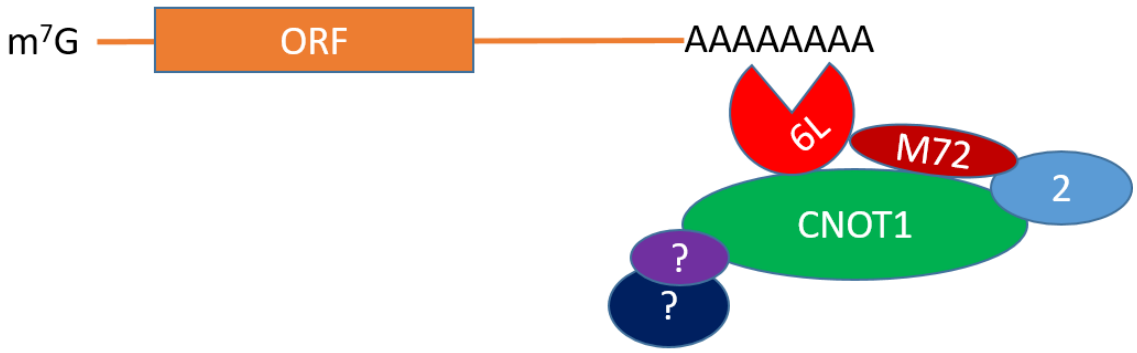

Figure 4.1: Hypothetical model for M72 mediated function.

Schematic illustrating current working model that M72 co-opts components of CCR4-NOT complex, specifically CNOT1, CNOT2, CNOT6L and possibly other unknown mediators. A hypothesis suggesting that M72 utilizes the CCR4-NOT complex components to turn over mRNA substrates is incorporated in the illustration.

4.3 Overall Conclusions

In conclusion, through my dissertation research, I have characterized a relatively unknown herpesvirus gene, MCMV M72, and revealed its contribution to virus replication not only in a cell culture system, but also in the natural host. I identified a complex expression profile for M72 protein. This opens up questions related to regulation of M72 and its isoforms. Other cytomegalovirus proteins also may be made during infection via internal methionine usage and it works as a viral strategy to expand its repertoire within the existing genomic context. I identified interactions of M72 with two novel host factors, TRiC/CCT and the CCR4-NOT complex. My work represents significant developments in the context of CMV host-pathogen interactions. Continued investigations into these novel host factors might expand our knowledge of drug targets against CMV and reveal intricate cellular mechanisms that viruses exploit.

Chapter 5: Materials and Methods

5.1 Chapter 2 Materials and Methods

5.1.1 Plasmids and Transfections

M72 expression constructs were generated by PCR amplification of nucleotides 104,289 to 103,084 using the MCMV K181 bacterial artificial chromosome (BAC) pARK25 (Accession No. AM886412.1) [192], as template. Amplicons were cloned into the *EcoRI* and *XbaI* sites of p3XFLAG-CMV10 or p3XFLAG-CMV14 expression constructs (Sigma-Aldrich) or the *EcoRI* and *KpnI* sites of pEGFP-C1 (Clontech). Transfections were performed using GenJet transfection reagent (SignaGen Laboratories) according to manufacturer instructions. HA-CCT expression constructs [223] were a kind gift from Dr. Kyong-Tai Kim (Pohang University of Science and Technology, Republic of Korea). FLAG-tagged MHV68 ORF54 and ORF54$^{H80A/D85N}$ expression constructs [110] were a kind gift from Drs. Ting-Ting Wu and Ren Sun (University of California at Los Angeles).

5.1.2 Cells and Reagents

STO (CRL-1503), HEK293T (ATCC CRL-3216), SVEC4-10 endothelial cells (ATCC CRL-2181) and RAW264.7 murine macrophages (ATCC TIB-71) were propagated in Dulbecco's modified Eagle's medium (DMEM - Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (FCS, Life Technologies, Inc.) and 1% penicillin-streptomycin-glutamine (PSG, Life Technologies, Inc.). NIH3T3 murine fibroblasts (ATCC CRL-1658) were propagated in DMEM containing 10% heat-inactivated bovine calf serum (BCS, Life Technologies) and 1% PSG. Bone marrow-derived macrophage (BMDM) cultures were generated as previously described [14]. Briefly, pooled bone

marrow cells from flushed tibias and femurs were harvested into Dulbecco's PBS, placed in culture for at least 18 hr. in DMEM containing 10% FBS, and then differentiated for 5–7 days in DMEM containing 20% FBS and 20% L929-conditioned medium. Phosphonoformic acid (PFA) was from Sigma-Aldrich.

5.1.3 Generation of recombinant viruses

BAC mutagenesis and diagnosis was performed by recombineering as previously described [224]. Briefly, *E. coli* DH10B cell containing pARK25 and pSIM6 [225] were grown to an OD600 of 0.4 to 0.6, recombination functions induced by incubation at 42^{o}C, and cells were made electrocompetent by multiple washes in ice cold water. The levansucrase (SacB) and kanamycin (Kan) genes were amplified from plasmid pTBE100 [224] with 60 nucleotide base pair overhangs corresponding to MCMV genomic sequences. PCRs were treated with *DpnI*, and amplicons gel purified then were used to electroporate induced bacteria. Kanamycin-resistant, sucrose-sensitive clones were selected and assessed for insertion and genomic integrity by PCR and RFLP analysis. M72.SK (primers; SG005, 5'-*ACGGGACGCCTGCACAACGTCGGAAGGCGTCGCGACCTCGAGGAACAAAAGCAG CAGCAC*AATTCGAGCTCGGTACCCGG-3' and SG006, 5'-*ATCACGATCTTGTTGACGGTCGTATCCGGCACCGAAGCGGCGACCGACACCGGTA CGGAGGCC*ATCCCGGGAAAAGTGCCACC-3': italic indicates viral sequence) deletes viral sequence between nucleotide 102,772 – 105,791 and M72Flag.SK (primers; FC001,

5’*GGTAAACGTAGTTTTCCTGAGTACACTAGACAAGAGGTAATCTCTCTAGG*AATTC GAGCTCGGTACCCGG-3’ and FC002, 5’-*ACCGCGCGGAAGAGAGAATGCGAAGCGGTCGAGACTCGTCGAACGAGGGC*ATCC CGGGAAAAGTGCCACC) deletes the M72 stop codon (nucleotides 103,083 – 103,086) and introduces an additional 2.9 kb of sequence. Specific mutations were introduced by a second round of recombineering with individual amplicons generated by overlap extension PCR. M72StopS (primers; SG007, 5'-ATGAAGGATCCCTTTAATATCTTCG<u>AC**T**A**GT**</u>ACGAACCTTCC-3' and SG015, 5’-GGAAGGTTC<u>GT**AC**T**A**</u>GTCGAAGATATTAAAGGGATCCTTCAT 3’; bold indicates nucleotide substitution, underline indicates diagnostic restriction enzyme recognition site) and M72StopN (primers; SG009, 5'-ATGAAGGATCCCTTTAATA<u>**G**CT**AGC**</u>ACGACGACGAACCTTCC-3' and SG016, 5’-GGAAGGTTCGTCGTCGT<u>**GCT**AG**C**</u>TATTAAAGGGATCCTTCAT-3’) contain an engineered stop codon and a *SpeI* (nt 104,124 – 104,127), or an *NheI* (nt 104,135-104,130) diagnostic restriction site, respectively. M72.3XFlag inserts three tandem FLAG epitopes at the C-terminal end of M72, and was constructed by recombineering with an amplicon generated by overlap extension PCR (primers; FC003, 5’-GGACGTGTAAGTGTGTGGATTGTTG-3’ and FC009, 5’-TGACCTAGAGAGATTACCTCTTGTCTAG using template pARK25; FC004, 5’-GATGGCCAAGATCATCTTCACGAC-3’ and FC010, 5’-CTAGACAAGAGGTAATCTCTCTAGGTCACTACTTGTCATCGTCATCCTTGTAG-

3’ template p3XFLAG-CMV-14-M72). Colonies were screened for kanamycin sensitivity and sucrose resistance, and positive clones were confirmed by PCR and RFLP analysis. PCR amplification and diagnostic restriction digest of M72 region confirmed the incorporation of the intended sequence in the M72 mutants. Infectious virus was reconstituted as previously described [224], amplified by growth in STO cells in the presence of 25 μg/ml 6-thioguanine (Sigma-Aldrich, St. Louis, MO), and plaque purified by limiting dilution. Parallel stocks were produced by infecting BALB/cJ mice with initial transfection supernatants of WT and M72Stop mutants (M72StopS and M72StopN). Infected salivary glands were harvested 14 days post infection (d.p.i.), sonicated, clarified and used to infect NIH3T3 fibroblasts. Viral stocks were generated, clarified, concentrated and titered by plaque assay as previously described on NIH3T3 fibroblasts [224]. All viral stocks were confirmed to be GFP negative, indicating excision of the BAC. All M72 viral stocks were confirmed by sequencing of the recombinant junctions, introduced mutations, and surrounding regions.

5.1.4 Infections, *in vitro* growth, and determination of viral titers

Viral titers were determined by plaque assays performed on NIH3T3 fibroblasts. Viral growth *in vitro* was determined by infection of indicated cell lines at a multiplicity of infection (MOI) of 5 PFU/cell to measure single step growth kinetics, or at 0.05 PFU/cell to measure multiple cycles of replication. Viruses were adsorbed for 2 hr at 37^{o}C in a volume of 0.4 ml. Cells and supernatants for quantitation were harvested at indicated times post infection, and frozen at -80^{o}C. Samples were subjected to one round of

freeze/thawing, and virus was quantitated by plaque assay on NIH3T3 fibroblasts as previously described [224]. Organs for virus quantitation were thawed and homogenized by sonication. Organ homogenates were serially diluted in complete media, and the titers were determined by plaque assay on NIH3T3 fibroblasts as previously described [224].

5.1.5 Immunoblotting

NIH3T3 fibroblasts were seeded in 35 mm dishes and infected with MCMV-M72.3XFlag recombinant virus at an MOI of 5 PFU/cell in the absence or presence of 200 µg/ml Phosphonoformic acid (PFA, Sigma). Whole cell lysates were made at the indicated time points by removing media, washing the cells with PBS, adding 200 µl of 1X SDS-lysis buffer directly on the cells and freezing the dish at -20ºC. All the samples were heated at 95ºC for 10 min, analyzed on 10% SDS-PAGE gel, immobilized on nitrocellulose membranes and probed with indicated antibodies.

5.1.6 Immunoprecipitation, Immunoblotting and Antibodies

Immunoprecipitations were performed by lysing transfected or infected cells in NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, and 1% NP-40, supplemented with c0mplete Mini EDTA free Protease inhibitor cocktail (Roche), Phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)) on ice for 20 min, and clarifying by high-speed centrifugation for 10 min. Supernatants were pre-cleared with Mouse IgG Agarose beads (Sigma), immunoprecipitated using anti-FLAG M2 affinity beads (Sigma), followed by elution with 20 µg of 3X FLAG peptide (Sigma). Samples were mixed with 2X- SDS sample buffer, boiled at 95ºC for 10 min, and resolved on 10% SDS-PAGE gels.

Immunoblotting was performed as previously described [224]. The following antibodies were used: mouse anti-FLAG M2-Peroxidase (Clone M2; Sigma-Aldrich), mouse anti-HA-peroxidase (Clone HA-7; Sigma-Aldrich), rabbit anti-GFP (FL) (sc-8334; Santa Cruz), mouse anti-β-actin (Clone AC-74; Sigma), mouse anti-m123/IE1 (CROMA101; Center for Proteomics, University of Rijeka), mouse anti-M112-113/E1 (CROMA103; Center for Proteomics, University of Rijeka), mouse anti-M55 (C1 M55.01; Center for Proteomics, University of Rijeka), rabbit anti-M86 (MCP – a gift from Dr. Laura Hanson, Texas Women's College, Denton, TX), rabbit TCP1/CCT1 polyclonal (10320-1-AP, Proteintech), rabbit CCT7 polyclonal (15994-1-AP, Proteintech), rabbit CCT8 polyclonal (12263-1-AP, Proteintech), donkey anti-mouse IgG-HRP (Vector Laboratories), and goat anti-rabbit IgG-HRP (Vector Laboratories). Blots were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and exposed to film. Digital images were generated with a CanoSCAN LIDE 700F slide/film scanner (Cannon) and images processed with Canvas X16 software (ACD Systems International, Ft. Lauderdale, DL, USA). No digital enhancements were applied.

5.1.7 Animal infections and organ harvest

BALB/cJ mice were obtained from the Jackson Laboratory. Animals were bred and maintained at the Animal Resources Center (ARC) at the University of Texas at Austin in accordance with the institutional guidelines. Male and female animals were used between 7 and 12 weeks of age. Intraperitoneal (i.p.) infections were performed by injection of 10^5 PFU in a volume of 0.5 ml. Upon sacrifice, organs were harvested by sterile dissection,

placed into 1 ml complete DMEM and frozen at -80^{o}C before titer determination. Each time point represents n = 10 or 15 mice per group. All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

5.1.8 dUTPase assay

dUTPase assays were performed as previously described [110]. Briefly, epitope tagged M72, MHV68 ORF54 or its catalytic mutant ORF54$^{H80A/D85N}$ were transfected into HEK293T cells. Cells were lysed 36 hr post transfection in NP-40 lysis buffer on ice for 20 min, and clarified by centrifugation. Supernatants were pre-cleared with Mouse IgG Agarose beads (Sigma), immunoprecipitated using anti-FLAG M2 affinity beads (Sigma), followed by elution with 20 µg of 3X FLAG peptide (Sigma). Five µl of purified FLAG-tagged proteins were incubated for indicated times at 37^{o}C with five µl of 5 mM dUTP (Promega) in ten µl of 2x reaction buffer (100 mM Tris pH 7.5, 20mM $MgCl_2$, 20 mM DTT, 0.2 mg/ml BSA). Reactions were terminated by freezing. PCR was performed using the dUTP sample, along with dATP, dCTP, and dGTP to amplify a small region of M72 expression plasmid DNA (primers; SG020 5'-CTTGAATTCAGCCACCATGGCGAAGCACAACAGAAGG-3' and SG032, 5'-CAATCTAGACTTCTCGGGACGACGCGCTTCC-3'). PCR cycle conditions were 95^{o}C, 2 min; 94^{o}C, 30 sec; 65^{o}C, 30 sec; 72^{0}C, 30 sec for 35 cycles, 72^{o}C 10 min and hold at 4^{o}C. PCR products were analyzed on 2% agarose gel.

5.1.9 Sample Preparation, LC-MS/MS, and Data Analysis

M72-3XFLAG or its corresponding empty vector control p3XFLAG-CMV-14 were transfected into NIH3T3 fibroblasts in 10 cm dishes. Whole cell lysates were collected 24 hr later in 1% NP-40 lysis buffer and subjected to FLAG-immunoprecipitation as described above. Samples were eluted using 50 μg 3X FLAG peptide (Sigma) for 30 min on an orbital rotator at room temperature. Eluted proteins were boiled in 1X SDS sample buffer, separated on a 10% SDS-PAGE gel, stained with 0.1% Coomassie-Blue dye and destained. Bands present in the M72-3XFLAG lane compared to the vector control were selected, and gel slices submitted for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Proteomics Core Facility, UT Austin). Gel bands were subjected to in-gel tryptic digest and separated by liquid chromatography on a Dionex Ultimate 3000 nanoflow UPCL system (Thermo Scientific). Eluting peptides were directly analyzed by nano-electrospray ionization-tandem mass spectrometry on a Thermo Orbitrap Elite (Thermo Scientific) instrument for a processing time of 1 hr. Protein identification was provided by the University of Texas at Austin Proteomics Facility following previously published procedures [226]. Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if greater than 57.0% probability to achieve an FDR less than 0.5% by the Peptide Prophet algorithm [6] with Scaffold delta-mass correction. Protein identifications were accepted if greater than 99.0% probability and contained at least 5 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [7]. Proteins that contained similar peptides

and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from gene_association.goa_uniprot (downloaded Jul 14, 2014) [12].

5.1.9 TRiC/CCT substrate assay

The substrate assay was adapted from a previously described assay [202]. Briefly, HEK293T cells were transfected with M72-3XFLAG. At 24 hr post transfection, cells were lysed in NP-40 lysis buffer on ice for 20 min, clarified by centrifugation for 10 min and supernatants were aliquoted into 4 parts. Samples were either mock treated or incubated with 15mM EDTA or 5mM $MgCl_2$ with or without 5mM ATP for 40 min at room temperature. Samples were pre-cleared with normal Rabbit-IgG (Santa Cruz Biotech) and ProteinA/G PLUS-Agarose beads (Santa Cruz Biotech). Samples were immunoprecipitated using a mixture of 0.5 µg of rabbit anti-CCT8 antibody and 0.5 µg of rabbit TCP1 antibody and Protein A/G agarose beads overnight at 4ºC on an orbital rotator. Beads were boiled in SDS sample buffer at 95ºC for 10 min, resolved on 10% SDS-PAGE and used for immunoblotting.

5.1.10 *in vitro* transcription/translation

Assays were performed as previously described [200]. Briefly, coupled *in vitro* transcription and translation reactions were conducted using the TNT-coupled rabbit reticulocyte lysate (RRL) system (Promega, L4610) according to the manufacturer's instructions. All open reading frame templates for *in vitro* transcription and translation were sub-cloned into the pcDNA3.1+ vector. All reactions were incubated at 30°C for

variable intervals depending on the experimental conditions. Reactions were supplemented with [^{35}S]-methionine (Perkin Elmer, NEG709A500UC) for radiolabeling and RNasin Plus RNase Inhibitor (N2611). Translation reactions were terminated by four-fold dilution in stop buffer (20 mM HEPES-KOH, pH 7.4, 100 mM potassium acetate, 5 mM magnesium acetate, 5 mM EDTA, 2 mM methionine, 1 mM DTT, 2 mM puromycin) unless otherwise specified. Samples were then resolved by native- and SDS-PAGE.

5.2 CHAPTER 3 MATERIALS AND METHODS

5.2.1 Plasmids and Transfections

M72 full-length and truncation expression constructs cloned into p3XFLAG-CMV14 and M72 full-length cloned into pEGFP-C1 were made as previously described [1]. Plasmid transfections were performed using GenJet transfection reagent (SignaGen Laboratories). pT7-EGFP-C1-Hs CNOT1 (# 37370), pT7-EGFP-C1-Hs CNOT2 (#37371) and pT7-EGFP-C1-Hs CNOT6L (# 37369) were obtained from Addgene.

5.2.2 Cells

HEK293T cells (ATCC CRL-3216) were propagated in Dulbecco's modified Eagle's medium (DMEM - Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (FCS, Life Technologies, Inc.) and 1% penicillin-streptomycin-glutamine (PSG, Life Technologies, Inc.). NIH3T3 murine fibroblasts (ATCC CRL-1658) were propagated in DMEM containing 10% heat-inactivated bovine calf serum (BCS, Life Technologies) and 1% PSG.

5.2.3 Viruses

Mouse-passaged viral stocks were titered and used for infections as previously described [1]. Briefly, the WT K181 MCMV containing bacterial artificial chromosome was utilized to generate M72StopS mutant virus by recombineering. The initial transfection supernatants of WT and M72StopS mutant were infected in BALB/cJ mice; salivary glands were harvested 14 days post infection. These organs were sonicated, and viral stocks generated on NIH3T3 cells.

5.2.4 siRNA treatment for depletion of CNOT1 and TAB182

ON-TARGETplus SMART pool Mouse CNOT1 and Mouse TAB182 and scrambled control were used (GE Dharmacon). NIH3T3 cells were seeded in 6-well tissue culture plates at $0.3x10^6$ cells per well. At 24 hr, media was changed to antibiotic-free DMEM and transfected with siRNAs diluted in Opti-MEM (Thermo Fisher Scientific) at concentration of 200 picomoles per µl siRNA per well in a 6-well plate using Lipofectamine RNAiMAX (ThermoFisher Scientific). After 24 hr, these siRNA transfected NIH3T3 cells were split and plated in 12-well tissue culture plates at $0.1x10^6$ cell per well, and used the following day for infection.

5.2.5 Infections, *in vitro* growth, and determination of viral titers

Indicated viruses were adsorbed for 2 hr at 37^0C in 0.4 ml volume with intermittent shaking. Infection of NIH3T3 cells was at 5 PFU/cell (high multiplicity of infection or high MOI) or at 0.05 PFU/cell (low multiplicity of infection or low MOI) as indicated. Cells and supernatants were harvested at the indicated times post infection and frozen at -80^0C. After one round of freeze/thawing, samples were quantitated on NIH3T3 cells by plaque assay.

5.2.6 Immunoblotting

NIH3T3 cells were mock or infected at multiplicity of infection 5 PFU/cell with indicated viruses. Cells were washed with PBS at the indicated time points and lysed by directly adding 200 µl of 1X SDS-lysis buffer, boiled at 95^0C for 10 min and analyzed on 4–20% Mini PROTEAN TGX SDS-PAGE gel (Bio Rad), transferred and immunoblotted as previously described [1].

5.2.7 Immunoprecipitations, Immunoblotting and Antibodies

Transfected and infected cells were lysed as previously described [1]. Briefly, NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, and 1% NP-40, supplemented with c0mplete Mini EDTA free Protease inhibitor cocktail (Roche), Phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)), was added, samples were incubated on ice for 20 min and clarified for 10 min by high-speed centrifugation. For immunoprecipitations, lysates were pre-cleared with mouse IgG agarose beads (Sigma) or rabbit protein IgG (Santa Cruz) and Protein A/G agarose beads (Santa Cruz). Subsequently they were incubated in anti-FLAG M2 affinity beads (Sigma) or anti-CNOT1 antibody (Proteintech) and Protein A/G agarose beads overnight at 4ºC, washed and beads were boiled in 1X-SDS lysis buffer at 95ºC for 10 min, and resolved on 10% SDS-PAGE gels. Immunoblotting was performed as previously described [224]. The following antibodies were used: mouse anti-FLAG M2-Peroxidase (Clone M2; Sigma-Aldrich), mouse anti-m123/IE1 (IE1.01; Center for Proteomics, University of Rijeka), mouse anti-β-actin (Clone AC-74; Sigma), rabbit anti-GFP (FL) (sc-8334; Santa Cruz), rabbit anti-CNOT1 (14276-1-AP; Proteintech), mouse anti-CNOT2 (sc-2191c2a; Santa Cruz), mouse anti-CNOT7 (sc-

101009; Santa Cruz), goat anti-TAB182 (N-20; sc-49261; Santa Cruz), donkey anti-mouse IgG-HRP (Vector Laboratories), goat anti-rabbit IgG-HRP (Vector Laboratories) and horse anti-goat IgG-HRP (Vector Laboratories). Blots were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and exposed to film. Digital images were generated with a CanoSCAN LIDE 700F slide/film scanner (Cannon) and images were processed with Canvas X16 software (ACD Systems International, Ft. Lauderdale, DL, USA). No digital enhancements were applied.

5.2.8 Sample Preparation, LC-MS/MS, and Data Analysis

This procedure was previously described [1]. Briefly, M72-3XFLAG or its corresponding empty vector control p3XFLAG-CMV-14 were transfected into NIH3T3 cells, immunoprecipitated with FLAG-specific antibody, and eluted with 3XFLAG peptide. Samples were separated on SDS-PAGE gels, stained with Coomassie-Blue dye, destained and enriched bands in M72-3XFLAG lane selected and subjected to LC-MS/MS analysis. Protein identification was provided by the Proteomics core facility at University of Texas at Austin utilizing the UniProt mouse reference genome.

REFERENCES

1. Gopal, S., et al., *Murine cytomegalovirus M72 promotes acute virus replication in vivo and is a substrate of the TRiC/CCT complex.* Virology, 2018. **522**: p. 92-105.
2. Davison, A.J., *Herpesvirus systematics.* Veterinary Microbiology, 2010. **143**(1-2): p. 52-69.
3. AJ., D., *Comparative analysis of the genomes.* , in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.*, C.-F.G. Arvin A, Mocarski E et. al., Editor. 2007, Cambridge University Press: Cambridge.
4. RJ, W., *Herpesviruses*, in *Medical Microbiology*, B. S., Editor. 1996: Galveston (TX): University of Texas Medical Branch at Galveston.
5. Brown, J.C. and W.W. Newcomb, *Herpesvirus Capsid Assembly: Insights from Structural Analysis.* Current opinion in virology, 2011. **1**(2): p. 142-149.
6. Grinde, B., *Herpesviruses: latency and reactivation – viral strategies and host response.* Journal of Oral Microbiology, 2013. **5**: p. 10.3402/jom.v5i0.22766.
7. Markowitz, J.A.S.F.X.M.R.S.G.L.A.F.K.L.A.J.N.G.M.M.M.E.L.L.E., *National Seroprevalence and Trends in Herpes Simplex Virus Type 1 in the United States, 1976–1994.* Sexually Transmitted Diseases, 2004. **31**: p. 753-760.
8. Berry, N.J., et al., *Seroepidemiologic studies on the acquisition of antibodies to cytomegalovirus, herpes simplex virus, and human immunodeficiency virus among general hospital patients and those attending a clinic for sexually transmitted diseases.* J Med Virol, 1988. **24**(4): p. 385-93.
9. Johnston, C., S.L. Gottlieb, and A. Wald, *Status of vaccine research and development of vaccines for herpes simplex virus.* Vaccine, 2016. **34**(26): p. 2948-2952.
10. Oxman, M.N., et al., *A Vaccine to Prevent Herpes Zoster and Postherpetic Neuralgia in Older Adults.* New England Journal of Medicine, 2005. **352**(22): p. 2271-2284.
11. C., E.V., *Human Herpesvirus Vaccines and Future Directions.* American Journal of Transplantation, 2013. **13**(s3): p. 79-86.
12. Cohen, J.I., *Epstein–barr virus vaccines.* Clinical & Translational Immunology, 2015. **4**(1): p. e32.
13. Katarzyna, K., et al., *Acyclovir in the Treatment of Herpes Viruses – A Review.* Current Medicinal Chemistry, 2018. **25**: p. 1-18.
14. Opstelten, W., et al., *Treatment of herpes zoster.* Canadian Family Physician, 2008. **54**(3): p. 373-377.
15. Moyle, G., et al., *Foscarnet and Ganciclovir in the treatment of CMV retinitis in AIDS patients: A randomised comparison.* Journal of Infection, 1992. **25**(1): p. 21-27.

16. Munawwar, A. and S. Singh, *Human herpesviruses as copathogens of HIV infection, Their role in HIV transmission, and disease progression.* Journal of Laboratory Physicians, 2016. **8**(1): p. 5-18.
17. Ho, M., *The history of cytomegalovirus and its diseases.* Medical Microbiology and Immunology, 2008. **197**(2): p. 65-73.
18. Lipschütz, B., *Untersuchungen über die Ätiologie der Krankheiten der Herpesgruppe (Herpes zoster, Herpes genitalis, Herpes febrilis).* Archiv für Dermatologie und Syphilis, 1921. **136**(3): p. 428-482.
19. VonGlahn, W.C. and A.M. Pappenheimer, *Intranuclear Inclusions in Visceral Disease.* The American Journal of Pathology, 1925. **1**(5): p. 445-466.3.
20. Enders, J.F., T.H. Weller, and F.C. Robbins, *Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues.* Science, 1949. **109**(2822): p. 85.
21. Smith, M.G., *Propagation in Tissue Cultures of a Cytopathogenic Virus from Human Salivary Gland Virus (SGV) Disease.* Proceedings of the Society for Experimental Biology and Medicine, 1956. **92**(2): p. 424-430.
22. Wallace, P.R., et al., *Cytopathogenic Agent Resembling Human Salivary Gland Virus Recovered from Tissue Cultures of Human Adenoids.* Proceedings of the Society for Experimental Biology and Medicine, 1956. **92**(2): p. 418-424.
23. Weller, T.H., et al., *Isolation of Intranuclear Inclusion Producing Agents from Infants with Illnesses Resembling Cytomegalic Inclusion Disease.* Proceedings of the Society for Experimental Biology and Medicine, 1957. **94**(1): p. 4-12.
24. Weller, T.H., *Review. Cytomegaloviruses: the difficult years.* J Infect Dis, 1970. **122**(6): p. 532-9.
25. Weller, T.H., J.B. Hanshaw, and D.M.E. Scott, *Serologic differentiation of viruses responsible for cytomegalic inclusion disease.* Virology, 1960. **12**(1): p. 130-132.
26. Schottstedt, V., et al., *Human Cytomegalovirus (HCMV) – Revised.* Transfusion Medicine and Hemotherapy, 2010. **37**(6): p. 365-375.
27. Chen, D.H., et al., *Three-Dimensional Visualization of Tegument/Capsid Interactions in the Intact Human Cytomegalovirus.* Virology, 1999. **260**(1): p. 10-16.
28. Baldick, C.J. and T. Shenk, *Proteins associated with purified human cytomegalovirus particles.* Journal of Virology, 1996. **70**(9): p. 6097-105.
29. Varnum, S.M., et al., *Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: the HCMV Proteome.* Journal of Virology, 2004. **78**(20): p. 10960-10966.
30. Gibson, W., *Structure and Formation of the Cytomegalovirus Virion*, in *Human Cytomegalovirus*, T.E. Shenk and M.F. Stinski, Editors. 2008, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 187-204.
31. Butcher, S.J., et al., *Structure of the Human Cytomegalovirus B Capsid by Electron Cryomicroscopy and Image Reconstruction.* Journal of Structural Biology, 1998. **124**(1): p. 70-76.

32. Sinzger, C., et al., *Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues.* Journal of General Virology, 1995. **76**(4): p. 741-750.
33. Wang, X., et al., *Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus.* Nature, 2003. **424**: p. 456.
34. Soroceanu, L., A. Akhavan, and C.S. Cobbs, *Platelet-derived growth factor-α receptor activation is required for human cytomegalovirus infection.* Nature, 2008. **455**: p. 391.
35. Viswanathan, K., et al., *BST2/Tetherin Enhances Entry of Human Cytomegalovirus.* PLOS Pathogens, 2011. **7**(11): p. e1002332.
36. Vanarsdall, A.L. and D.C. Johnson, *Human cytomegalovirus entry into cells.* Current opinion in virology, 2012. **2**(1): p. 10.1016/j.coviro.2012.01.001.
37. Ryckman, B.J., et al., *Human Cytomegalovirus Entry into Epithelial and Endothelial Cells Depends on Genes UL128 to UL150 and Occurs by Endocytosis and Low-pH Fusion.* Journal of Virology, 2006. **80**(2): p. 710-722.
38. Compton, T., R.R. Nepomuceno, and D.M. Nowlin, *Human cytomegalovirus penetrates host cells by PH-independent fusion at the cell surface.* Virology, 1992. **191**(1): p. 387-395.
39. Isaacson, M.K. and T. Compton, *Human Cytomegalovirus Glycoprotein B Is Required for Virus Entry and Cell-to-Cell Spread but Not for Virion Attachment, Assembly, or Egress.* Journal of Virology, 2009. **83**(8): p. 3891-3903.
40. Mach, M., et al., *Complex Formation by Human Cytomegalovirus Glycoproteins M (gpUL100) and N (gpUL73).* Journal of Virology, 2000. **74**(24): p. 11881-11892.
41. Bowman, J.J., et al., *Rhesus and Human Cytomegalovirus Glycoprotein L Are Required for Infection and Cell-to-Cell Spread of Virus but Cannot Complement Each Other.* Journal of Virology, 2011. **85**(5): p. 2089-2099.
42. Huber, M.T. and T. Compton, *The Human Cytomegalovirus UL74 Gene Encodes the Third Component of the Glycoprotein H-Glycoprotein L-Containing Envelope Complex.* Journal of Virology, 1998. **72**(10): p. 8191-8197.
43. Cha, T.A., et al., *Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains.* Journal of Virology, 1996. **70**(1): p. 78-83.
44. Hahn, G., et al., *Human Cytomegalovirus UL131-128 Genes Are Indispensable for Virus Growth in Endothelial Cells and Virus Transfer to Leukocytes.* Journal of Virology, 2004. **78**(18): p. 10023-10033.
45. Wang, D. and T. Shenk, *Human Cytomegalovirus UL131 Open Reading Frame Is Required for Epithelial Cell Tropism.* Journal of Virology, 2005. **79**(16): p. 10330-10338.
46. Kalejta, R.F., *Functions of Human Cytomegalovirus Tegument Proteins Prior to Immediate Early Gene Expression*, in *Human Cytomegalovirus*, T.E. Shenk and M.F. Stinski, Editors. 2008, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 101-115.

47. Ogawa-Goto, K., et al., *Microtubule Network Facilitates Nuclear Targeting of Human Cytomegalovirus Capsid.* Journal of Virology, 2003. **77**(15): p. 8541-8547.
48. Chambers, J., et al., *DNA Microarrays of the Complex Human Cytomegalovirus Genome: Profiling Kinetic Class with Drug Sensitivity of Viral Gene Expression.* Journal of Virology, 1999. **73**(7): p. 5757-5766.
49. Mocarski E, S.T., Griffiths P, R.F., P. Knipe DM, editor. , *Fields Virology*. 2013.
50. Das, S. and P.E. Pellett, *Spatial Relationships between Markers for Secretory and Endosomal Machinery in Human Cytomegalovirus-Infected Cells versus Those in Uninfected Cells.* Journal of Virology, 2011. **85**(12): p. 5864-5879.
51. Das, S., A. Vasanji, and P.E. Pellett, *Three-Dimensional Structure of the Human Cytomegalovirus Cytoplasmic Virion Assembly Complex Includes a Reoriented Secretory Apparatus.* Journal of Virology, 2007. **81**(21): p. 11861-11869.
52. Moorman, N.J., et al., *A Targeted Spatial-Temporal Proteomics Approach Implicates Multiple Cellular Trafficking Pathways in Human Cytomegalovirus Virion Maturation.* Molecular & Cellular Proteomics : MCP, 2010. **9**(5): p. 851-860.
53. Alwine, J.C., *The Human Cytomegalovirus Assembly Compartment: A Masterpiece of Viral Manipulation of Cellular Processes That Facilitates Assembly and Egress.* PLoS Pathogens, 2012. **8**(9): p. e1002878.
54. Beltran, P.M.J. and I.M. Cristea, *The life cycle and pathogenesis of human cytomegalovirus infection: lessons from proteomics.* Expert review of proteomics, 2014. **11**(6): p. 697-711.
55. Rafailidis, P.I., et al., *Severe cytomegalovirus infection in apparently immunocompetent patients: a systematic review.* Virology Journal, 2008. **5**: p. 47-47.
56. Daniel Lancini, H.M.F., Robert Flower and Chris Hogan, *Cytomegalovirus disease in immunocompetent adults.* The Medical Journal of Australia, 2014. **201**(10): p. 578-580.
57. Riou, R., et al., *Severe symptomatic primary HCMV infection despite effective innate and adaptive immune responses.* Journal of Virology, 2016.
58. Pass, R.F., et al., *Young Children as a Probable Source of Maternal and Congenital Cytomegalovirus Infection.* New England Journal of Medicine, 1987. **316**(22): p. 1366-1370.
59. Pass, R.F., et al., *Cytomegalovirus Infection in a Day-Care Center.* New England Journal of Medicine, 1982. **307**(8): p. 477-479.
60. Boppana, S.B., et al., *Intrauterine Transmission of Cytomegalovirus to Infants of Women with Preconceptional Immunity.* New England Journal of Medicine, 2001. **344**(18): p. 1366-1371.
61. Louise McCormick, A. and E.S. Mocarski, *The immunological underpinnings of vaccinations to prevent cytomegalovirus disease.* Cellular And Molecular Immunology, 2014. **12**: p. 170.

62. Azevedo*, L.S., et al., *Cytomegalovirus infection in transplant recipients.* Clinics, 2015. **70**(7): p. 515-523.
63. Rubin, R.H., *The indirect effects of cytomegalovirus infection on the outcome of organ transplantation.* JAMA, 1989. **261**(24): p. 3607-3609.
64. Limaye, A.P., et al., *Emergence of ganciclovir-resistant cytomegalovirus disease among recipients of solid-organ transplants.* The Lancet, 2000. **356**(9230): p. 645-649.
65. Jabs, D.A., *Cytomegalovirus Retinitis and the Acquired Immunodeficiency Syndrome—Bench to Bedside: LXVII Edward Jackson Memorial Lecture.* American Journal of Ophthalmology, 2011. **151**(2): p. 198-216.e1.
66. Deayton, J.R., et al., *Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy.* The Lancet, 2004. **363**(9427): p. 2116-2121.
67. Kempen, J.H., et al., *Mortality Risk for Patients with Cytomegalovirus Retinitis and Acquired Immune Deficiency Syndrome.* Clinical Infectious Diseases, 2003. **37**(10): p. 1365-1373.
68. Stagno, S., et al., *Congenital Cytomegalovirus Infection.* New England Journal of Medicine, 1977. **296**(22): p. 1254-1258.
69. D., G.P., C.-B. A., and H.R. B., *A prospective study of primary cytomegalovirus infection in pregnant women.* BJOG: An International Journal of Obstetrics & Gynaecology, 1980. **87**(4): p. 308-314.
70. J., C.M., S.D. Scott, and H.T. B., *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection.* Reviews in Medical Virology, 2010. **20**(4): p. 202-213.
71. C., D.S., G.S. D., and R.D. S., *New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection.* Reviews in Medical Virology, 2007. **17**(5): p. 355-363.
72. Adland, E., et al., *Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era.* Frontiers in Microbiology, 2015. **6**: p. 1016.
73. Razonable, R.R., *Cytomegalovirus infection after liver transplantation: Current concepts and challenges.* World Journal of Gastroenterology : WJG, 2008. **14**(31): p. 4849-4860.
74. KB, B.S.a.F., *Persistence in the population: epidemiology and transmisson*, in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*, C.-F.G.a.M.E. Arvin A, Editor. 2007, Cambridge University Press: Cambridge.
75. Miller, L.W., et al., *Infection after heart transplantation: a multiinstitutional study. Cardiac Transplant Research Database Group.* J Heart Lung Transplant, 1994. **13**(3): p. 381-92; discussion 393.
76. Wang, C., et al., *Attribution of Congenital Cytomegalovirus Infection to Primary Versus Non-Primary Maternal Infection.* Clinical Infectious Diseases, 2011. **52**(2): p. e11-e13.

77. Griffiths, P., et al., *Desirability and feasibility of a vaccine against cytomegalovirus.* Vaccine, 2013. **31**: p. B197-B203.
78. Lanzieri, T.M., et al., *Systematic review of the birth prevalence of congenital cytomegalovirus infection in developing countries.* International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases, 2014. **22**: p. 44-48.
79. Manicklal, S., et al., *The "Silent" Global Burden of Congenital Cytomegalovirus.* Clinical Microbiology Reviews, 2013. **26**(1): p. 86-102.
80. Staczek, J., *Animal cytomegaloviruses.* Microbiological Reviews, 1990. **54**(3): p. 247-265.
81. Schleiss, M.R. and M.A. McVoy, *Guinea Pig Cytomegalovirus (GPCMV): A Model for the Study of the Prevention and Treatment of Maternal-Fetal Transmission.* Future virology, 2010. **5**(2): p. 207-217.
82. Vink, C., E. Beuken, and C.A. Bruggeman, *Complete DNA sequence of the rat cytomegalovirus genome.* J Virol, 2000. **74**(16): p. 7656-65.
83. Streblow, D.N., et al., *Rat cytomegalovirus-accelerated transplant vascular sclerosis is reduced with mutation of the chemokine-receptor R33.* Am J Transplant, 2005. **5**(3): p. 436-42.
84. Streblow, D.N., et al., *Mechanisms of Cytomegalovirus-Accelerated Vascular Disease: Induction of Paracrine Factors That Promote Angiogenesis and Wound Healing.* Current topics in microbiology and immunology, 2008. **325**: p. 397-415.
85. Fryer, J.F., et al., *Susceptibility of porcine cytomegalovirus to antiviral drugs.* J Antimicrob Chemother, 2004. **53**(6): p. 975-80.
86. Davison, A.J., et al., *The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome.* J Gen Virol, 2003. **84**(Pt 1): p. 17-28.
87. Powers, C. and K. Früh, *Rhesus CMV: an emerging animal model for human CMV.* Medical microbiology and immunology, 2008. **197**(2): p. 109-115.
88. R. Shellam, G., et al., *Chapter 1 - Murine Cytomegalovirus and Other Herpesviruses*, in *The Mouse in Biomedical Research (Second Edition)*, J.G. Fox, et al., Editors. 2007, Academic Press: Burlington. p. 1-48.
89. Qin, W., et al., *Efficient CRISPR/Cas9-Mediated Genome Editing in Mice by Zygote Electroporation of Nuclease.* Genetics, 2015. **200**(2): p. 423-30.
90. Medearis, D.N., Jr., *Mouse cytomegalovirus infection. II. Observations during prolonged infections.* Am J Hyg, 1964. **80**: p. 103-12.
91. Crawford, L.B., et al., *Humanized Mouse Models of Human Cytomegalovirus Infection.* Current opinion in virology, 2015. **13**: p. 86-92.
92. Harris, J.M., E.M. McIntosh, and G.E. Muscat, *Structure/function analysis of a dUTPase: catalytic mechanism of a potential chemotherapeutic target.* J Mol Biol, 1999. **288**(2): p. 275-87.

93. Vertessy, B.G. and J. Toth, *Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases.* Acc Chem Res, 2009. **42**(1): p. 97-106.
94. Baldo, A.M. and M.A. McClure, *Evolution and Horizontal Transfer of dUTPase-Encoding Genes in Viruses and Their Hosts.* Journal of Virology, 1999. **73**(9): p. 7710-7721.
95. Traut, T.W., *Physiological concentrations of purines and pyrimidines.* Mol Cell Biochem, 1994. **140**(1): p. 1-22.
96. Diamond, T.L., et al., *Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase.* J Biol Chem, 2004. **279**(49): p. 51545-53.
97. Ladner, R.D. and S.J. Caradonna, *The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species.* J Biol Chem, 1997. **272**(30): p. 19072-80.
98. Hizi, A. and E. Herzig, *dUTPase: the frequently overlooked enzyme encoded by many retroviruses.* Retrovirology, 2015. **12**(1): p. 70.
99. Lerner, D.L., et al., *Increased mutation frequency of feline immunodeficiency virus lacking functional deoxyuridine-triphosphatase.* Proceedings of the National Academy of Sciences, 1995. **92**(16): p. 7480-7484.
100. Davison, A.J. and N.D. Stow, *New genes from old: redeployment of dUTPase by herpesviruses.* J Virol, 2005. **79**(20): p. 12880-92.
101. Fisher, F.B. and V.G. Preston, *Isolation and characterisation of herpes simplex virus type 1 mutants which fail to induce dUTPase activity.* Virology, 1986. **148**(1): p. 190-197.
102. Pyles, R.B., N.M. Sawtell, and R.L. Thompson, *Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency.* Journal of Virology, 1992. **66**(11): p. 6706-6713.
103. Zhang, R., et al., *Pseudorabies Virus dUTPase UL50 Induces Lysosomal Degradation of Type I Interferon Receptor 1 and Antagonizes the Alpha Interferon Response.* J Virol, 2017. **91**(21).
104. Ross, J., M. Williams, and J.I. Cohen, *Disruption of the Varicella–Zoster Virus dUTPase and the Adjacent ORF9A Gene Results in Impaired Growth and Reduced Syncytia Formationin Vitro.* Virology, 1997. **234**(2): p. 186-195.
105. Ward, T.M., et al., *The simian varicella virus uracil DNA glycosylase and dUTPase genes are expressed in vivo, but are non-essential for replication in cell culture.* Virus Research, 2009. **142**(1-2): p. 78-84.
106. Caposio, P., et al., *Evidence that the human cytomegalovirus 46-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasts.* Virology, 2004. **325**(2): p. 264-76.

107. Ariza, M.-E., et al., *The EBV-Encoded dUTPase Activates NF-κB through the TLR2 and MyD88-Dependent Signaling Pathway.* The Journal of Immunology, 2009. **182**(2): p. 851-859.
108. Ariza, M.E., et al., *Epstein-Barr Virus Encoded dUTPase Containing Exosomes Modulate Innate and Adaptive Immune Responses in Human Dendritic Cells and Peripheral Blood Mononuclear Cells.* PLOS ONE, 2013. **8**(7): p. e69827.
109. Madrid, A.S. and D. Ganem, *Kaposi's Sarcoma-Associated Herpesvirus ORF54/dUTPase Downregulates a Ligand for the NK Activating Receptor NKp44.* Journal of Virology, 2012. **86**(16): p. 8693-8704.
110. Leang, R.S., et al., *The Anti-interferon Activity of Conserved Viral dUTPase ORF54 is Essential for an Effective MHV-68 Infection.* PLOS Pathogens, 2011. **7**(10): p. e1002292.
111. Ariza, M.E., R. Glaser, and M.V. Williams, *Human herpesviruses-encoded dUTPases: a family of proteins that modulate dendritic cell function and innate immunity.* Frontiers in Microbiology, 2014. **5**(504).
112. Williams, M., B. Cox, and M. Ariza, *Herpesviruses dUTPases: A New Family of Pathogen-Associated Molecular Pattern (PAMP) Proteins with Implications for Human Disease.* Pathogens, 2017. **6**(1): p. 2.
113. Hartl, F.U., *Molecular chaperones in cellular protein folding.* Nature, 1996. **381**(6583): p. 571-9.
114. Saibil, H., *Chaperone machines for protein folding, unfolding and disaggregation.* Nature reviews. Molecular cell biology, 2013. **14**(10): p. 630-642.
115. Evstigneeva, Z.G., N.A. Solov'eva, and L.I. Sidel'nikova, *Structures and Functions of Chaperones and Chaperonins (Review).* Applied Biochemistry and Microbiology, 2001. **37**(1): p. 1-13.
116. Kubota, H., et al., *Identification of six Tcp-1-related genes encoding divergent subunits of the TCP-1-containing chaperonin.* Curr Biol, 1994. **4**(2): p. 89-99.
117. Kubota, H., et al., *Tissue-specific subunit of the mouse cytosolic chaperonin-containing TCP-1.* FEBS Lett, 1997. **402**(1): p. 53-6.
118. Meyer, A.S., et al., *Closing the Folding Chamber of the Eukaryotic Chaperonin Requires the Transition State of ATP Hydrolysis.* Cell, 2003. **113**(3): p. 369-381.
119. Llorca, O., et al., *ATP Binding Induces Large Conformational Changes in the Apical and Equatorial Domains of the Eukaryotic Chaperonin Containing TCP-1 Complex.* Journal of Biological Chemistry, 1998. **273**(17): p. 10091-10094.
120. Dekker, C., et al., *The crystal structure of yeast CCT reveals intrinsic asymmetry of eukaryotic cytosolic chaperonins.* The EMBO Journal, 2011. **30**(15): p. 3078-3090.
121. Kalisman, N., C.M. Adams, and M. Levitt, *Subunit order of eukaryotic TRiC/CCT chaperonin by cross-linking, mass spectrometry, and combinatorial homology modeling.* Proceedings of the National Academy of Sciences, 2012. **109**(8): p. 2884-2889.

122. Kim, S., K.R. Willison, and A.L. Horwich, *Cystosolic chaperonin subunits have a conserved ATPase domain but diverged polypeptide-binding domains.* Trends in Biochemical Sciences, 1994. **19**(12): p. 543-548.
123. Llorca, O., et al., *Eukaryotic type II chaperonin CCT interacts with actin through specific subunits.* Nature, 1999. **402**: p. 693.
124. Gomez-Puertas, P., et al., *The substrate recognition mechanisms in chaperonins.* J Mol Recognit, 2004. **17**(2): p. 85-94.
125. Horwich, A.L., et al., *Two Families of Chaperonin: Physiology and Mechanism.* Annual Review of Cell and Developmental Biology, 2007. **23**(1): p. 115-145.
126. Yam, A.Y., et al., *Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly-made proteins with complex topologies.* Nature structural & molecular biology, 2008. **15**(12): p. 1255-1262.
127. Hynes, G.M. and K.R. Willison, *Individual subunits of the eukaryotic cytosolic chaperonin mediate interactions with binding sites located on subdomains of beta-actin.* J Biol Chem, 2000. **275**(25): p. 18985-94.
128. Pappenberger, G., et al., *Crystal structure of the CCTgamma apical domain: implications for substrate binding to the eukaryotic cytosolic chaperonin.* J Mol Biol, 2002. **318**(5): p. 1367-79.
129. Joachimiak, L.A., et al., *The structural basis of substrate recognition by the eukaryotic chaperonin TRiC/CCT.* Cell, 2014. **159**(5): p. 1042-1055.
130. Gao, Y., et al., *A cytoplasmic chaperonin that catalyzes beta-actin folding.* Cell, 1992. **69**(6): p. 1043-50.
131. Frydman, J., et al., *Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits.* The EMBO Journal, 1992. **11**(13): p. 4767-4778.
132. Srikakulam, R. and D.A. Winkelmann, *Myosin II folding is mediated by a molecular chaperonin.* J Biol Chem, 1999. **274**(38): p. 27265-73.
133. Won, K.A., et al., *Maturation of human cyclin E requires the function of eukaryotic chaperonin CCT.* Mol Cell Biol, 1998. **18**(12): p. 7584-9.
134. Liu, X., et al., *CCT Chaperonin Complex Is Required for the Biogenesis of Functional Plk1.* Molecular and Cellular Biology, 2005. **25**(12): p. 4993-5010.
135. Li, D. and R. Roberts, *WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases.* Cell Mol Life Sci, 2001. **58**(14): p. 2085-97.
136. Camasses, A., et al., *The CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20.* Mol Cell, 2003. **12**(1): p. 87-100.
137. Feldman, D.E., et al., *Formation of the VHL-elongin BC tumor suppressor complex is mediated by the chaperonin TRiC.* Mol Cell, 1999. **4**(6): p. 1051-61.
138. Melville, M.W., et al., *The Hsp70 and TRiC/CCT chaperone systems cooperate in vivo to assemble the von Hippel-Lindau tumor suppressor complex.* Mol Cell Biol, 2003. **23**(9): p. 3141-51.

139. Zhang, J., et al., *Cellular Chaperonin CCTγ Contributes to Rabies Virus Replication during Infection.* Journal of Virology, 2013. **87**(13): p. 7608-7621.
140. Inoue, Y., et al., *Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein.* Virology, 2011. **410**(1): p. 38-47.
141. Lingappa, J.R., et al., *A eukaryotic cytosolic chaperonin is associated with a high molecular weight intermediate in the assembly of hepatitis B virus capsid, a multimeric particle.* J Cell Biol, 1994. **125**(1): p. 99-111.
142. Hong, S., et al., *Type D retrovirus Gag polyprotein interacts with the cytosolic chaperonin TRiC.* J Virol, 2001. **75**(6): p. 2526-34.
143. Knowlton, J.J., et al., *The TRiC chaperonin controls reovirus replication through outer-capsid folding.* Nat Microbiol, 2018. **3**(4): p. 481-493.
144. Kashuba, E., et al., *Epstein-Barr virus-encoded nuclear protein EBNA-3 interacts with the epsilon-subunit of the T-complex protein 1 chaperonin complex.* J Hum Virol, 1999. **2**(1): p. 33-7.
145. Draper, M.P., C. Salvadore, and C.L. Denis, *Identification of a mouse protein whose homolog in Saccharomyces cerevisiae is a component of the CCR4 transcriptional regulatory complex.* Molecular and Cellular Biology, 1995. **15**(7): p. 3487-95.
146. Albert, T.K., et al., *Isolation and characterization of human orthologs of yeast CCR4–NOT complex subunits.* Nucleic Acids Research, 2000. **28**(3): p. 809-817.
147. Xu, K., et al., *Insights into the structure and architecture of the CCR4-NOT complex.* Front Genet, 2014. **5**: p. 137.
148. Miller, J.E. and J.C. Reese, *Ccr4-Not complex: the control freak of eukaryotic cells.* Critical Reviews in Biochemistry and Molecular Biology, 2012. **47**(4): p. 315-333.
149. N.C., L., *Composition and function of Ccr4-Not protein complexes* 2010, Utrecht University: The Netherlands. p. 124.
150. Mauxion, F., B. Prève, and B. Séraphin, *C2ORF29/CNOT11 and CNOT10 form a new module of the CCR4-NOT complex.* RNA Biology, 2013. **10**(2): p. 267-276.
151. Lau, N.-C., et al., *Human Ccr4–Not complexes contain variable deadenylase subunits.* Biochemical Journal, 2009. **422**(3): p. 443-453.
152. Chen, C., et al., *Distinct expression patterns of the subunits of the CCR4–NOT deadenylase complex during neural development.* Biochemical and Biophysical Research Communications, 2011. **411**(2): p. 360-364.
153. Collart, M.A., *The Ccr4-Not complex is a key regulator of eukaryotic gene expression.* Wiley Interdiscip Rev RNA, 2016. **7**(4): p. 438-54.
154. Shirai, Y.-T., et al., *Multifunctional roles of the mammalian CCR4–NOT complex in physiological phenomena.* Frontiers in Genetics, 2014. **5**: p. 286.
155. Denis, C.L. and J. Chen, *The CCR4-NOT complex plays diverse roles in mRNA metabolism.* Prog Nucleic Acid Res Mol Biol, 2003. **73**: p. 221-50.
156. Ito, K., et al., *The role of the CNOT1 subunit of the CCR4-NOT complex in mRNA deadenylation and cell viability.* Protein Cell, 2011. **2**(9): p. 755-63.

157. Fabian, M.R., et al., *Structural basis for the recruitment of the human CCR4-NOT deadenylase complex by tristetraprolin.* Nat Struct Mol Biol, 2013. **20**(6): p. 735-9.
158. Bhandari, D., et al., *Structural basis for the Nanos-mediated recruitment of the CCR4-NOT complex and translational repression.* Genes Dev, 2014. **28**(8): p. 888-901.
159. Zwartjes, C.G., et al., *Repression of promoter activity by CNOT2, a subunit of the transcription regulatory Ccr4-not complex.* J Biol Chem, 2004. **279**(12): p. 10848-54.
160. Faraji, F., et al., *An integrated systems genetics screen reveals the transcriptional structure of inherited predisposition to metastatic disease.* Genome Res, 2014. **24**(2): p. 227-40.
161. Jeong, K., et al., *CNOT2 promotes degradation of p62/SQSTM1 as a negative regulator in ATG5 dependent autophagy.* Oncotarget, 2017. **8**(28): p. 46034-46046.
162. Panasenko, O.O., *The role of the E3 ligase Not4 in cotranslational quality control.* Front Genet, 2014. **5**: p. 141.
163. Winkler, G.S. and D.L. Balacco, *Heterogeneity and complexity within the nuclease module of the Ccr4-Not complex.* Front Genet, 2013. **4**: p. 296.
164. Mittal, S., et al., *The Ccr4a (CNOT6) and Ccr4b (CNOT6L) deadenylase subunits of the human Ccr4–Not complex contribute to the prevention of cell death and senescence.* Molecular Biology of the Cell, 2011. **22**(6): p. 748-758.
165. Morita, M., et al., *Depletion of mammalian CCR4b deadenylase triggers elevation of the p27Kip1 mRNA level and impairs cell growth.* Mol Cell Biol, 2007. **27**(13): p. 4980-90.
166. Berthet, C., et al., *CCR4-Associated Factor CAF1 Is an Essential Factor for Spermatogenesis.* Molecular and Cellular Biology, 2004. **24**(13): p. 5808-5820.
167. Washio-Oikawa, K., et al., *Cnot7-null mice exhibit high bone mass phenotype and modulation of BMP actions.* J Bone Miner Res, 2007. **22**(8): p. 1217-23.
168. Seimiya, H. and S. Smith, *The Telomeric Poly(ADP-ribose) Polymerase, Tankyrase 1, Contains Multiple Binding Sites for Telomeric Repeat Binding Factor 1 (TRF1) and a Novel Acceptor, 182-kDa Tankyrase-binding Protein (TAB182).* Journal of Biological Chemistry, 2002. **277**(16): p. 14116-14126.
169. Smith, S., et al., *Tankyrase, a Poly(ADP-Ribose) Polymerase at Human Telomeres.* Science, 1998. **282**(5393): p. 1484-1487.
170. Vyas, S., et al., *A systematic analysis of the PARP protein family identifies new functions critical for cell physiology.* Nature Communications, 2013. **4**: p. 2240.
171. Matsuoka, S., et al., *ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage.* Science, 2007. **316**(5828): p. 1160-1166.
172. Zou, L.-H., et al., *TNKS1BP1 functions in DNA double-strand break repair though facilitating DNA-PKcs autophosphorylation dependent on PARP-1.* Oncotarget, 2015. **6**(9): p. 7011-7022.

173. Tan, W., et al., *Overexpression of TNKS1BP1 in lung cancers and its involvement in homologous recombination pathway of DNA double-strand breaks.* Cancer Medicine, 2017. **6**(2): p. 483-493.
174. Ohishi, T., et al., *Tankyrase-Binding Protein TNKS1BP1 Regulates Actin Cytoskeleton Rearrangement and Cancer Cell Invasion.* Cancer Research, 2017. **77**(9): p. 2328-2338.
175. Hutchins, J.R., et al., *Systematic analysis of human protein complexes identifies chromosome segregation proteins.* Science, 2010. **328**(5978): p. 593-9.
176. Havugimana, P.C., et al., *A census of human soluble protein complexes.* Cell, 2012. **150**(5): p. 1068-81.
177. Huttlin, E.L., et al., *The BioPlex Network: A Systematic Exploration of the Human Interactome.* Cell, 2015. **162**(2): p. 425-440.
178. Hagkarim, N.C., et al., *Degradation of a novel DNA damage response protein, tankyrase 1 binding protein 1 (Tab182), following adenovirus infection.* J Virol, 2018.
179. White, E.A., et al., *Comprehensive Analysis of Host Cellular Interactions with Human Papillomavirus E6 Proteins Identifies New E6 Binding Partners and Reflects Viral Diversity.* Journal of Virology, 2012. **86**(24): p. 13174-13186.
180. Li, Q., et al., *A genome-wide genetic screen for host factors required for hepatitis C virus propagation.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(38): p. 16410-16415.
181. Lin, Y.-C., K.-S. Jeng, and M.M.C. Lai, *CNOT4-Mediated Ubiquitination of Influenza A Virus Nucleoprotein Promotes Viral RNA Replication.* mBio, 2017. **8**(3).
182. Griffiths, P.D., *Burden of disease associated with human cytomegalovirus and prospects for elimination by universal immunisation.* The Lancet Infectious Diseases, 2012. **12**(10): p. 790-798.
183. Boppana, S.B., S.A. Ross, and K.B. Fowler, *Congenital Cytomegalovirus Infection: Clinical Outcome.* Clinical Infectious Diseases, 2013. **57**(suppl_4): p. S178-S181.
184. Stratton KR, D.J., Lawrence RS, eds, *Vaccines for the 21st Century: A Tool for Decisionmaking*, ed. K.R. Stratton, J.S. Durch, and R.S. Lawrence. 2000, Washington, DC: The National Academies Press. 476.
185. Plosa, E.J., et al., *Cytomegalovirus Infection.* Pediatrics in Review, 2012. **33**(4): p. 156-163.
186. Ramanan, P. and R.R. Razonable, *Cytomegalovirus Infections in Solid Organ Transplantation: A Review.* Infection & Chemotherapy, 2013. **45**(3): p. 260-271.
187. Barry, P.A., et al., *Nonhuman Primate Models of Intrauterine Cytomegalovirus Infection.* ILAR Journal, 2006. **47**(1): p. 49-64.
188. Schleiss, M.R., *Nonprimate Models of Congenital Cytomegalovirus (CMV) Infection: Gaining Insight into Pathogenesis and Prevention of Disease in Newborns.* ILAR Journal, 2006. **47**(1): p. 65-72.

189. Smith, C. and R. Khanna, *The Development of Prophylactic and Therapeutic EBV Vaccines*, in *Epstein Barr Virus Volume 2: One Herpes Virus: Many Diseases*, C. Münz, Editor. 2015, Springer International Publishing: Cham. p. 455-473.
190. Ward, T.M., et al., *The simian varicella virus uracil DNA glycosylase and dUTPase genes are expressed in vivo, but are non-essential for replication in cell culture.* Virus Res, 2009. **142**(1-2): p. 78-84.
191. Huang, E.-S. and R.A. Johnson, *Human cytomegalovirus – no longer just a DNA virus.* Nature Medicine, 2000. **6**: p. 863.
192. Redwood, A.J., et al., *Use of a Murine Cytomegalovirus K181-Derived Bacterial Artificial Chromosome as a Vaccine Vector for Immunocontraception.* Journal of Virology, 2005. **79**(5): p. 2998-3008.
193. Juranic Lisnic, V., et al., *Dual Analysis of the Murine Cytomegalovirus and Host Cell Transcriptomes Reveal New Aspects of the Virus-Host Cell Interface.* PLOS Pathogens, 2013. **9**(9): p. e1003611.
194. Rapp, M., et al., *Expression of the murine cytomegalovirus glycoprotein H by recombinant vaccinia virus.* Journal of General Virology, 1994. **75**(1): p. 183-188.
195. Scalzo, A.A., et al., *The murine cytomegalovirus M73.5 gene, a member of a 3′ co-terminal alternatively spliced gene family, encodes the gp24 virion glycoprotein.* Virology, 2004. **329**(2): p. 234-250.
196. Kattenhorn, L.M., et al., *Identification of Proteins Associated with Murine Cytomegalovirus Virions.* Journal of Virology, 2004. **78**(20): p. 11187-11197.
197. Lopez, T., K. Dalton, and J. Frydman, *The mechanism and function of group II chaperonins.* Journal of molecular biology, 2015. **427**(18): p. 2919-2930.
198. Rüßmann, F., et al., *Folding of large multidomain proteins by partial encapsulation in the chaperonin TRiC/CCT.* Proceedings of the National Academy of Sciences, 2012. **109**(52): p. 21208-21215.
199. Fislová, T., et al., *Association of the Influenza Virus RNA Polymerase Subunit PB2 with the Host Chaperonin CCT.* Journal of Virology, 2010. **84**(17): p. 8691-8699.
200. Knowlton, J.J., et al., *The TRiC chaperonin controls reovirus replication through outer-capsid folding.* Nature Microbiology, 2018. **3**(4): p. 481-493.
201. Gao, Y., et al., *A cytoplasmic chaperonin that catalyzes β-actin folding.* Cell, 1992. **69**(6): p. 1043-1050.
202. Won, K.-A., et al., *Maturation of Human Cyclin E Requires the Function of Eukaryotic Chaperonin CCT.* Molecular and Cellular Biology, 1998. **18**(12): p. 7584-7589.
203. Kasembeli, M., et al., *Modulation of STAT3 Folding and Function by TRiC/CCT Chaperonin.* PLOS Biology, 2014. **12**(4): p. e1001844.
204. Freund, A., et al., *Proteostatic control of telomerase function through TRiC-mediated folding of TCAB1.* Cell, 2014. **159**(6): p. 1389-403.
205. Readhead, B., et al., *Multiscale Analysis of Independent Alzheimer’s Cohorts Finds Disruption of Molecular, Genetic, and Clinical Networks by Human Herpesvirus.* Neuron.

206. John, E.M., W.D. Nigel, and J.M. Duncan, *Evolution of the dUTPase Gene of Mammalian and Avian Herpesviruses.* Current Protein & Peptide Science, 2001. **2**(4): p. 325-333.
207. Dunn, W., et al., *Functional profiling of a human cytomegalovirus genome.* Proceedings of the National Academy of Sciences, 2003. **100**(24): p. 14223.
208. Yu, D., M.C. Silva, and T. Shenk, *Functional map of human cytomegalovirus AD169 defined by global mutational analysis.* Proceedings of the National Academy of Sciences, 2003. **100**(21): p. 12396.
209. Stoddart, C.A., et al., *Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus.* Journal of Virology, 1994. **68**(10): p. 6243-6253.
210. Caviness, K., et al., *Complex Interplay of the UL136 Isoforms Balances Cytomegalovirus Replication and Latency.* mBio, 2016. **7**(2).
211. Caviness, K., et al., *Complex Expression of the UL136 Gene of Human Cytomegalovirus Results in Multiple Protein Isoforms with Unique Roles in Replication.* Journal of Virology, 2014. **88**(24): p. 14412-14425.
212. Umashankar, M., et al., *A Novel Human Cytomegalovirus Locus Modulates Cell Type-Specific Outcomes of Infection.* PLOS Pathogens, 2011. **7**(12): p. e1002444.
213. Fossum, E., et al., *Evolutionarily Conserved Herpesviral Protein Interaction Networks.* PLOS Pathogens, 2009. **5**(9): p. e1000570.
214. Wang, H., et al., *Crystal structure of the human CNOT6L nuclease domain reveals strict poly(A) substrate specificity.* EMBO J, 2010. **29**(15): p. 2566-76.
215. Tay, Y., et al., *Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs.* Cell, 2011. **147**(2): p. 344-57.
216. Smith, H.R.C., et al., *Recognition of a virus-encoded ligand by a natural killer cell activation receptor.* Proceedings of the National Academy of Sciences, 2002. **99**(13): p. 8826.
217. Miyasaka, T., et al., *Interaction of antiproliferative protein Tob with the CCR4-NOT deadenylase complex.* Cancer Sci, 2008. **99**(4): p. 755-61.
218. Sacher, T., et al., *The role of cell types in cytomegalovirus infection in vivo.* European Journal of Cell Biology, 2012. **91**(1): p. 70-77.
219. Brameier, M., A. Krings, and R.M. MacCallum, *NucPred--predicting nuclear localization of proteins.* Bioinformatics, 2007. **23**(9): p. 1159-60.
220. Pavel, M., et al., *CCT complex restricts neuropathogenic protein aggregation via autophagy.* Nat Commun, 2016. **7**: p. 13821.
221. Carr, A.C., et al., *Targeting chaperonin containing TCP1 (CCT) as a molecular therapeutic for small cell lung cancer.* Oncotarget, 2017. **8**(66): p. 110273-110288.
222. Iwasaki, T., W.W. Chin, and L. Ko, *Identification and characterization of RRM-containing coactivator activator (CoAA) as TRBP-interacting protein, and its splice variant as a coactivator modulator (CoAM).* J Biol Chem, 2001. **276**(36): p. 33375-83.

223. Kim, S., et al., *Vaccinia-Related Kinase 2 Mediates Accumulation of Polyglutamine Aggregates via Negative Regulation of the Chaperonin TRiC.* Molecular and Cellular Biology, 2014. **34**(4): p. 643-652.
224. Upton, J.W., W.J. Kaiser, and E.S. Mocarski, *Virus Inhibition of RIP3-Dependent Necrosis.* Cell Host & Microbe, 2010. **7**(4): p. 302-313.
225. Datta, S., N. Costantino, and D.L. Court, *A set of recombineering plasmids for gram-negative bacteria.* Gene, 2006. **379**: p. 109-115.
226. Knauf, G.A., et al., *Exploring the Antimicrobial Action of Quaternary Amines against Acinetobacter baumannii.* mBio, 2018. **9**(1).